







NATIONAL INSTITUTES OF HEALTH  
NIH LIBRARY

FEB 2 1995

BLOG 10, 10 CENTER DR.  
BETHESDA, MD 20892-1150





# LABORATORY OF INFECTIOUS DISEASES

1994 Annual Report

## Table of Contents

<b>Z01-AI</b>		
<b>Project Number</b>		<b>Page</b>
Summary		9-1
	<b>RESPIRATORY VIRUSES SECTION</b>	
00323-13	Parainfluenza Virus Type 3 (PIV3) Gene Expression -- Collins	9-21
00324-13	Laboratory Studies of Influenza Viruses -- Murphy	9-22
00325-13	Study of Respiratory Viruses in Primates -- Murphy	9-23
00326-13	Study of Respiratory Viruses in Volunteers -- Murphy	9-24
00327-13	Laboratory Studies of Parainfluenza Type 3 Virus -- Hall	9-25
00345-13	Laboratory Studies of Respiratory Syncytial Virus -- Murphy	9-26
00368-12	Structural Analysis of the Genome of Respiratory Syncytial Virus (RSV) -- Collins	9-27
00372-12	Roles of RSV Proteins in Host Immunity and Molecular Approaches to Vaccine Design -- Collins	9-28
00498-08	Synthesis, Processing and Functions of the Proteins of Human RSV -- Collins	9-29
	<b>HEPATITIS VIRUSES SECTION</b>	
00308-07	<i>In Vitro</i> and <i>In Vivo</i> Studies of Hepatitis A Virus -- Purcell (Inactive)	9-30
00311-13	Search for New Hepatitis Agents -- Purcell	9-31
00314-13	Woodchuck Virus: Molecular Biological Studies -- Miller	9-32
00530-07	Molecular and Computer Analysis of the Hepatitis B Virus Genome -- Miller	9-33



00569-05	Determination of Genetic Markers of Virulence and Adaptation to Cell-Culture of Hepatitis A Virus -- Emerson	9-34
00570-05	Molecular Biology of Hepatitis C Virus -- Miller	9-35
00596-04	<i>In Vivo</i> and <i>In Vitro</i> Studies of Hepatitis E Virus -- Emerson	9-36

## MOLECULAR VIRAL BIOLOGY SECTION

00500-06	Processing and Immunogenicity of Dengue Type 4 Virus Nonstructural Protein NS1 -- Lai (Inactive)	9-37
00531-05	Functional Analysis of Dengue Nonstructural Proteins NS2B and NS3 -- Lai (Inactive)	9-38
00571-03	Amino Acid Substitution at the NS1-NS2A Cleavage Junction of Dengue Virus Polyprotein -- Pethel (Inactive)	9-39
00572-03	Processing of Dengue Virus Polyprotein NS3-NS4A-NS4B-NS5 -- Lai (Inactive)	9-40
00598-02	Dengue Type 4 Virus Mutants Restricted in Polyprotein Processing -- Kawano (Inactive)	9-41
00600-04	Engineering Viable Dengue Virus 3' Noncoding Region Deletion Mutants for Use in Vaccines -- Men	9-42
00637-03	Development of New Approaches to Vaccines Against the Tick-Borne Encephalitis Virus Complex -- Pletnev	9-43
00638-02	Expression of Dengue and Other Flavivirus Proteins Using Baculovirus as a Vector -- Lai (Terminated)	9-44
00681-02	Determination of Genetic Loci of Dengue Type 2 Virus Neurovirulence in Mice -- Bray	9-45
00682-02	Evaluation of Intertypic Chimeric Dengue Viruses as Candidate Vaccines -- Bray	9-46
00683-02	Genetic Determinants of Dengue Virus Mouse Neurovirulence -- Lai	9-47
00706-01	Genetic Loci Responsible for Growth Restriction of Mouse-Adapted Dengue Viruses -- Lai	9-48





## EPIDEMIOLOGY SECTION

00333-13	Longitudinal and Cross-Sectional Studies of Viral Gastroenteritis in Infants and Young Children -- Kapikian	9-49
00339-13	Isolation and Serotypic Characterization of Human and Animal Rotaviruses -- Hoshino	9-50
00340-13	Genetic Studies of Rotavirus Pathogenesis -- Hoshino	9-51
00341-13	Evaluation of Live Attenuated Rotavirus Vaccines -- Kapikian	9-52
00342-13	Studies of Gastroenteritis Viruses by Electron Microscopy -- Kapikian	9-53
00343-13	The Role of Norwalk Virus and Related Norwalk-Like Viruses in Viral Gastroenteritis -- Green	9-54
00446-09	Testing of Rotavirus Vaccine Candidates in Venezuela and Peru -- Flores (Terminated)	9-55
00451-08	Immunological and Molecular Characterization of Rotavirus Antigens -- Kapikian (Terminated)	9-56
00507-07	Molecular Characterization of Rotavirus Serotypes -- Green (Inactive)	9-57
00533-06	Analysis of Rotavirus Proteins with Monoclonal Antibodies -- Green (Inactive)	9-58
00534-06	Molecular Analysis of Non-Group A Rotaviruses -- Green (Inactive)	9-59
00573-04	Expression of Rotavirus Proteins in <i>Salmonella</i> Bacteria -- Green (Inactive)	9-60
00574-04	Expression and Distribution of Conserved and Serotype-Specific Epitopes on Rotavirus VP8 -- Gorziglia (Terminated)	9-61
00576-04	Serotype Analysis and Characterization of Rotaviruses from Malaysia -- Rasool (Terminated)	9-62
00601-02	Development of Recombinant BCG-VP8 Vaccines -- Gorziglia (Terminated)	9-63
00602-02	Expression of Rotavirus Outer Capsid Protein VP4 by Recombinant-Adenovirus -- Gorziglia (Terminated)	9-64





00603-03	Monoclonal Antibodies Directed to VP8 Subunit of VP4 -- Gorziglia (Terminated)	9-65
00604-04	Cold-Adaptation of Human Rotaviruses -- Hoshino	9-66
00605-04	Experimental Infection of Chimpanzees by Human Rotavirus -- Kapikian	9-67
00639-02	Molecular Epidemiology of Rotaviruses -- Flores (Terminated)	9-68
00640-02	Synthetic Analogs of Rotavirus Genomic RNA Expressing a Foreign Marker Gene -- Gorziglia (Terminated)	9-69
00641-03	Analysis of Neutralizing Epitopes on Rotavirus Outer Capsid Proteins (VP4 and VP7) -- Hoshino	9-70
00642-03	Molecular Characterization of Norwalk and Norwalk- Like Viruses (27nm Viruses) -- Lew	9-71
00643-02	Expression of Rotavirus Outer Capsid Protein VP4 by a Baculovirus Recombinant -- Gorziglia (Terminated)	9-72
00684-02	Genetic and Serological Characterization of Equine Rotaviruses -- Ostlund	9-73
00685-02	Genetic Studies of the Cold-Adapted Human Rotavirus Phenotype -- Ostlund	9-74
00707-01	Identification of Genetic Determinants Required for the Replication of Caliciviruses -- Green	9-75

#### **IMMUNODEFICIENCY VIRUSES SECTION**

00370-12	Pathogenesis Studies of Simian Immunodeficiency Virus (SIV) Infection of Macaques -- Hirsch	9-76
00686-02	Vaccine Studies of Simian Immunodeficiency Virus (SIV) Infection of Macaques -- Hirsch	9-77



**Summary Statement**  
**LABORATORY OF INFECTIOUS DISEASES**  
**National Institute of Allergy and Infectious Diseases**  
**October 1, 1993 to September 30, 1994**

1993-1994 was a banner year for many of the LID research programs. Considerable progress was made in increasing our understanding of the molecular genetics, molecular virology, immunology and pathogenesis of viruses which are of major importance in serious acute respiratory tract disease, acute gastroenteritis of infancy and early childhood, hepatitis, the acute immunodeficiency syndrome (AIDS) and systemic flavivirus disease. As in the past, much of this basic knowledge has direct applicability to the development of new or improved strategies for prevention or treatment of these viral diseases that represent major targets for public health intervention. As a consequence of insights into these viruses and their diseases gained in LID during the past few years and continuing through 1993-1994, we have been able to accelerate the development of experimental live and inactivated virus vaccines and/or immunotherapy which show promise for prevention or therapy. It should be noted that these viruses are among the leading causes of infectious disease morbidity and mortality world-wide. Examples of significant advances include: (i) a live attenuated rotavirus vaccine which is approaching licensure; (ii) development of two live vaccine candidates for human parainfluenza virus type 3 (human PIV3) that have shown promise in phase I clinical trials in susceptible young children; (iii) promising live attenuated respiratory syncytial virus (RSV), candidate vaccines which are now in early phase I clinical trials in young children; (iv) cloned human monoclonal RSV antibody Fab fragments that rapidly clear pulmonary RSV in mice when administered directly into the lungs at the height of infection; (v) an inactivated hepatitis A virus (HAV) vaccine that is licensed in Europe and is awaiting licensure in the United States; (vi) a newly developed prototype recombinant hepatitis E virus (HEV) vaccine which protects monkeys against experimental challenge with the virus; and (vii) validation or advanced development of molecular strategies for constructing live attenuated vaccine strains of negative strand RNA viruses such as influenza A virus, RSV or human PIV3 by introducing site-specific attenuating mutations into viral cDNA.

**RESPIRATORY VIRUSES SECTION**

**Influenza A Virus: Molecular Strategy for Vaccine Construction.** Influenza A virus remains a very important cause of morbidity and mortality world-wide. The inactivated, vaccine provides significant protection (protective efficacy ~70-80%), however there is room for improvement in efficacy as well as our ability to respond rapidly to emergence of new antigenic variants in nature, *i.e.*, major drift or pandemic shift. Last year a new strategy was developed for the construction of live attenuated influenza A virus vaccines. Attenuating mutations were introduced into a cDNA copy of the influenza viral PB2 gene by site-directed mutagenesis and the mutant PB2 gene was subsequently rescued into an infectious virus. A *ts* mutation was chosen for introduction into viral cDNA because such mutations were known to be useful for attenuating viruses that infect the respiratory tract. The PB2 gene was selected for this strategy because it codes for an internal viral structural protein, a subunit of the viral polymerase, that is not a major protective antigen. Hence, it can be transferred to new epidemic or pandemic strains of influenza A virus without affecting the protective efficacy of vaccine against the new influenza A virus. The PB2 gene was also chosen for introduction of attenuating mutations because strong selective pressure for rescue of this transfecting gene could be applied by using a host-range restricted helper virus that was unable to replicate efficiently in mammalian cells unless its avian





influenza A virus PB2 gene was replaced by the corresponding gene of a human influenza A virus. Thus, the desired reassortant influenza A virus bearing the transfected mutant PB2 gene was easily recovered although it represented a very small proportion of the virus population. Initially, a plasmid was constructed that contained the PB2 gene of the human influenza A/Ann Arbor/6/60 wild type (*wt*) virus in which ser was substituted for asp at amino acid residue 265; this mutation had previously been shown to specify the *ts* and attenuation (*att*) phenotypes. Mutant PB2 vRNA, transcribed *in vitro* from cDNA, was transfected into primary chick cells in the presence of the host-range restricted helper virus that grew efficiently in avian cells, but not in mammalian cells. Subsequently, the desired transfectant virus was recovered in mammalian MDCK cells which exerted very strong selection for this virus. The transfectant virus possessed a human influenza A virus PB2 gene bearing the asp → ser mutation and as a consequence it exhibited the *ts* and *att* phenotypes. This transfectant virus was immunogenic and protected hamsters from subsequent challenge with *wt* virus. Thus, the feasibility of this new method for constructing attenuating influenza A virus genes was confirmed by the rescue of a mutant PB2 gene into a reassortant virus that exhibited the *att* phenotype and that induced a protective immune response.

Previously, we had identified 10 separate sites in the human influenza A virus PB2 gene at which a single viable *ts* mutation can occur. Three of these amino acid substitutions were introduced separately as a single mutation into the influenza A/AA/60 *wt* PB2 gene. Each of the transfectant viruses bearing a single distinct PB2 mutation exhibited the *ts* and *att* phenotypes and this confirmed our expectation that the introduced mutations would function in the PB2 gene. After confirming that the individual mutations were attenuating in the influenza A/AA/60 virus background, a double mutant was constructed that had a mutation at position 265 and position 556. This double mutant was more *ts* and more attenuated than either single mutant bearing only the 265 or 556 mutation.

**Respiratory Syncytial Virus (RSV).** This virus is the single most important cause of serious viral lower respiratory tract disease in infants and children, worldwide. In fact, RSV causes more severe lower tract disease in infants and young children than any other virus or group of related viruses. Previous attempts to develop a safe and effective RSV vaccine had all met with failure. A formalin-inactivated virus vaccine tested in the mid-1960s not only failed to protect against RSV infection or disease but caused a potentiation of disease when infection occurred. After the failure of the formalin-inactivated RSV vaccine, efforts to develop immunoprophylaxis were directed toward the selection of attenuated RSV mutants that could be used in a live vaccine administered topically. This decision was based, in part, on the observation that under normal circumstances potentiation of disease does not occur during reinfection with RSV.

Initially, cold-passaged (*cp*) or temperature-sensitive (*ts*) mutants of RSV were developed and shown to be attenuated and immunogenic. Nonetheless, early trials in susceptible infants and young children were somewhat discouraging: certain candidate vaccine strains, such as *cp*-RSV and the *ts*-1 mutant, were underattenuated, whereas another *ts* mutant, *ts*-2, was overattenuated. In addition, the *ts*-1 mutant demonstrated genetic instability that allowed it to lose its *ts* phenotype during replication *in vivo*. However, disease potentiation did not occur when vaccinees were reinfected with naturally circulating RSV. Recently, in an attempt to produce mutants free of these deficiencies, the *cp*-RSV mutant was used as a substrate for introduction of additional mutations. *cp*-RSV, which was derived from a subgroup A virus strain, was chosen because it had been shown to be attenuated for the lower respiratory tract of infants and young children and because it exhibited a marked restriction in its replication in the lungs of fully susceptible chimpanzees, which constitute the most relevant experimental host for the virus. *cp*-RSV does not exhibit the *ts* phenotype; its attenuation results from the acquisition of host-range



mutations. Mutants of *cp*-RSV generated by chemical mutagenesis exhibited a varying degree of temperature sensitivity and additional restriction of virus replication *in vivo*. Two of the more restricted mutants were subjected to another cycle of chemical mutagenesis which yielded mutants that were more temperature-sensitive and more restricted in replication in the nasal turbinates and lungs of mice as well as the nasopharynx of fully susceptible chimpanzees. Two distinct lineages of twice mutagenized *cp*-RSV have each yielded promising candidate live virus vaccine strains. In fully susceptible chimpanzees these strains exhibited: (i) complete or almost complete restriction of virus replication in the lungs; (ii) reduced replication in the nasopharynx, compared to *cp*-RSV, and almost complete attenuation of symptoms in this site; (iii) a significant increase in stability of the *ts* phenotype; and (iv) moderate immunogenicity. Despite reduced replication in chimpanzees, these further attenuated mutants induced almost complete protection against infection by wild type RSV.

The effect of passively-acquired serum RSV neutralizing antibodies on replication, immunogenicity and protective efficacy of these candidate live vaccine mutants was then evaluated because one month old infants, which are the target population for immunization, possess a moderate level of maternally-derived RSV neutralizing antibodies in their serum. Four to 6-week old infants usually have a serum titer of ~1:200. Significantly, chimpanzees which were infused with RSV neutralizing antibodies to achieve a serum titer of ~1:200 also responded to immunization by developing complete resistance in their lungs to subsequent challenge with wild type RSV. To our surprise, these animals developed an unusually high neutralizing antibody response following challenge with wild type RSV despite the marked restriction of replication of the challenge virus. We do not understand the basis for this unexpected priming effect of immunization that was not observed in the non-infused chimpanzees, but it certainly bodes well for the success of immunization with an attenuated RSV mutant that is administered in a two dose schedule early in infancy.

Efforts to produce a RSV subgroup B virus vaccine were initiated using the same approach that was found successful for the subgroup A mutants. Subgroup B wild type RSV (designated strain B1) was cold-passaged 52 times in simian Vero cells at low temperature (20-25°C) and virus was subjected to plaque purification at passages 19 and 52. One clone, derived by plaque purification, designated *cp*-RSV B1/2B5, was chosen for further analysis because it was highly attenuated in the upper and lower respiratory tract of the cotton rat. Evaluation of several clones of the *cp*-RSV B1 virus at different passage levels indicated that the *cp*-RSV B1/2B5 mutant had sustained three mutations that independently contributed to its attenuation phenotype. The presence of multiple mutations contributing to the attenuation phenotype was confirmed by demonstrating that the *cp*-RSV B1/2B5 mutant retained its attenuation phenotype following prolonged replication in immunosuppressed cotton rats. Importantly, RSV subgroup A and B mutants failed to interfere with each other *in vitro* or *in vivo* suggesting that it should be possible to formulate a live bivalent attenuated RSV vaccine.

**RSV: Strategy for Construction of Vaccine Strains by Introduction of Site-Specific Attenuating Mutations into the Viral Genome.** For many DNA and RNA viruses it is possible to introduce mutations into cloned viral DNA or cDNA and thereby into the viral genome. This capability makes it possible to create defined mutants for basic studies and vaccine development. However, this has not yet been possible for any of the nonsegmented negative strand RNA viruses. One reason is that homologous recombination apparently does not occur. More, importantly, the genome is not infectious as naked RNA. Instead, the minimal unit of infectivity is thought to be the transcriptionally-active nucleocapsid. Despite these obstacles, methods for producing infectious RSV from cDNA-encoded RNA are currently being pursued using helper-dependent cDNA-encoded "mini-genomes". This strategy was adopted so that the control signals for replication, transcription and packaging of viral RNA could be identified by





mutational analysis and subsequently utilized to achieve rescue of successively larger cDNA-encoded viral RNA constructs.

The prototypic RSV mini-genome, RSV-CAT, is a version of genomic RNA in which all of the viral genes were deleted and replaced by the foreign chloramphenicol acetyl transferase (CAT) marker gene. RSV-CAT contains the 3'-terminal 86 nucleotides of genomic RNA (including the leader region and gene-start and upstream nontranslated region of the NS1 gene), followed by the CAT orf, followed by the 5'-terminal 179 nucleotides of genomic RNA, including the downstream nontranslated and gene-end sequence of the L gene and the trailer region. Rescue was achieved when negative sense RSV-CAT RNA was synthesized from cDNA *in vitro* and then transfected into cells which had been infected with RSV to provide a source of viral proteins. Later experiments showed that positive sense RSV-CAT RNA could also be rescued efficiently. RSV-CAT was active in all of the stages of the RSV replicative cycle: replication, transcription of a subgenomic CAT-encoding polyadenylated mRNA, and packaging of the mini-genome into transmissible viral particles. Upon serial passage, RSV-CAT competed efficiently with the standard helper virus as evidenced by continued high levels of expression. In later experiments, rescue of RSV-CAT RNA was achieved with a mini-genome that contained only 105 nucleotides from the leader, NS-1 gene start signal, L gene end signal and a highly truncated trailer region. Thus, the segments from each end of genomic RNA represented in this shorter version of RSV-CAT appear to contain the *cis*-acting sequences necessary for all of these activities. This provides the first system for directly manipulating RSV genomic RNA for the study of transcription and RNA replication. In addition, progress has been made toward rescue of full-length viral RNA transcribed from cDNA. For example, dicistronic RSV minigenomes have been rescued. In one instance, the dicistronic construct contained the full-length RSV L gene sequence which represents 43% of the entire viral genome.

**Parainfluenza Virus Type 3 (PIV3): Vaccine Development.** PIV3, an enveloped RNA-containing paramyxovirus, is second only to RSV as a major etiologic agent of serious pediatric viral respiratory tract disease. Two approaches to vaccine development are currently being pursued with encouraging results. The first involves the use of a bovine PIV3 (BPIV3) that is antigenically related to human PIV3 (HPIV3) but attenuated for humans. In addition, infection of cotton rats or monkeys with BPIV3 induced resistance to subsequent challenge infection with HPIV3. It was felt that BPIV3, which had been selected over a long period of time for efficient replication in cows, should possess divergent sequences that restrict its replication in humans in a fashion analogous to that of cowpox virus which was much less virulent in humans than variola virus but, nonetheless, provided protection against smallpox. This approach to immunization against viral pathogens has been termed the "Jennerian" approach. The replication of two different bovine strains of PIV3 was restricted 100- to 1,000-fold in Old World primates (rhesus monkeys or chimpanzees) but was sufficient to induce high levels of serum neutralizing antibodies to HPIV3. The combined properties of restriction of replication and induction of a protective immune response to HPIV3 in non-human primates made the BPIV3 a promising candidate for use as a live-virus vaccine.

During the past year, early Phase 1 trials in seronegative vaccinees older than 6 months of age have been completed, and the results are encouraging. The vaccine was satisfactorily attenuated; illness occurred with equal frequency in both placebo controls and vaccinees. The attenuation phenotype of the virus was stable following replication in the vaccinees. Currently, the BPIV3 vaccine is being given to infants less than six months of age. This is the target population for this vaccine because HPIV3 causes serious disease during the first six months of life. Preliminary data for 8 young infant vaccinees indicates that the virus replicates to the same level as in older infants and is well tolerated.



The other approach involves the use of a cold-passaged HPIV3 mutant originally isolated by Dr. Robert Belshe (St. Louis University), an alumnus of LID. Currently, the mutant selected after 45 cold passages (*cp45* mutant) is being administered to seronegative infants and children in a collaborative study involving LID/NIAID, Johns Hopkins University, St. Louis University, and Vanderbilt University. The *cp45* mutant has been found to be satisfactorily attenuated, immunogenic, stable genetically, and immunogenic in seronegative subjects. Significantly, the *ts* phenotype of this virus was found to be very stable following prolonged replication in these susceptible subjects. Lederle-Praxis and LID/NIAID are planning to enter into a CRADA for the further development of this promising candidate vaccine.

**PIV3: Molecular Approaches to Vaccine Development.** Recently, LID scientists developed a new genetic system, namely the rescue system for minigenomes, which will allow experimental manipulations that were not previously possible for any of the nonsegmented negative strand viruses. This system is also being used to characterize *cis*-acting signals in the PIV3 genome.

A cDNA was constructed that encodes PIV3-CAT, a negative-sense RNA truncated version of PIV3 genome that contains the CAT reporter gene in place of the viral genes. Transfection of the PIV3-CAT RNA genome into PIV3-infected cells resulted in the expression of CAT. The PIV3 helper was strictly required. Activity was undiminished by treatment of the transfecting nucleic acid with DNase or by treatment of the cells with actinomycin D, consistent with the idea that expression was driven by the PIV3 polymerase acting on RNA. Passage of the supernatant fluids onto fresh cells 24 hours later resulted in the expression of CAT, indicating that the synthetic genome was incorporated into a transmissible material which resembled a virus particle in being sensitive to neutralizing antibody directed against the PIV3 HN protein. A second version of PIV3-CAT DNA was constructed to encode PIV3-CAT RNA as a positive-sense strand, which would correspond to the predicted replicative intermediate. The positive-sense version of PIV3-CAT was approximately one-fifth as active in the rescue assay as was the negative-sense version. The PIV3-CAT system has been used successfully to map *cis*-acting sequences in the terminal non-coding regions of the viral genome, identify the 3' promoter, and characterize the function of intergenic sequences.

A full-length cDNA copy of human PIV3 has been prepared and will be used in an attempt to rescue infectious virus from cDNA. The success in rescuing PIV3-CAT demonstrates that a cDNA-encoded genome, indeed, can be rendered biologically active. The finding that replicative intermediate RNA can be rescued suggests that this might be the optimal way of introducing a full-length RNA into cells for rescue; a naked negative-sense copy might be inactivated by hybridization to viral mRNAs before it can be encapsidated. The PIV3-CAT system also will be useful for mapping and evaluating attenuating mutations in live vaccine PIV3 strains which are under evaluation as potential live vaccines.

**Passive Prophylaxis or Therapy of RSV Disease Using Cloned Human RSV Monoclonal Antibodies.** Previously, we had shown that prevention of RSV infection in experimental animals could be achieved by passive transfer of RSV neutralizing antibodies. This approach was recently validated by others in a clinical trial involving high risk infants. Monthly intravenous inoculation of human IgG prepared from pooled plasma containing a high titer of RSV neutralizing antibodies was recently shown to be effective in prophylaxis of serious RSV disease. However, this is not a practical strategy because of the large quantity of globulin required for each dose (0.75 gm per kg) and the need to administer this material intravenously in the clinic or hospital over a 2 to 6 hour interval every month during the fall, winter and early spring.



Earlier studies that we performed in cotton rats indicated that RSV antibodies were also effective for therapy of RSV infection, suggesting that a similar approach might also prove to be useful for therapy of serious RSV disease in infants and young children.

The large amount of human IgG (derived from pooled plasma) required for effective prophylaxis or therapy of RSV infection makes it unlikely that appropriate clinical realization of these approaches can be achieved with currently available human IgG preparations. RSV disease in high-risk young infants and children and adults illustrates the need for solution of this problem. There is a relatively large population of approximately 250,000 infants, children and adults in the US at high risk of developing severe or fatal RSV illness who would benefit from passive RSV immunoprophylaxis, especially since a vaccine is not available at this time. The high risk population includes premature infants, infants and children with bronchopulmonary dysplasia, congenital heart disease, cystic fibrosis, cancer or various forms of immunodeficiency as well as adults who are immunodeficient incident to organ transplantation or HIV infection.

Routine use of passive immunotherapy for the treatment of serious RSV disease will also require the development of human IgG preparations with higher RSV specific activity which can be administered rapidly by the intravenous, intramuscular or aerosol route. Recently, in order to meet the needs just described, a Scripps Research Institute-NIAID collaborative effort was initiated to clone human RSV monoclonal antibodies that neutralize the virus with high efficiency and exhibit strong protective and therapeutic efficacy *in vivo*.

Recombinant human respiratory syncytial virus (RSV) monoclonal antibody Fabs were identified by antigen selection from random combinatorial libraries displayed at the tip of filamentous phage. Two such Fabs, which exhibited high binding affinity for RSV F glycoprotein (a major protective antigen), were evaluated for therapeutic efficacy in infected mice just before or at the time of peak virus replication in the lungs. Fab 19, which neutralized RSV infectivity with high efficiency in tissue culture, was effective therapeutically when delivered directly into the lungs by intranasal instillation under anesthesia. In contrast, RSV Fab 126, which failed to neutralize virus in cell culture, did not exhibit a therapeutic effect under these conditions. The amount of Fab 19 required to effect a 5,000- to 12,000-fold reduction in titer of RSV in the lungs within 24 hr was rather small. In four separate experiments, a single instillation of 12.9 to 50  $\mu$ g of RSV Fab 19 was sufficient to achieve such a reduction in pulmonary virus in a 25g mouse. The use of Fabs instead of the whole immunoglobulin molecules from which they are derived reduced the protein content of a therapeutic dose. This is important because the protein load that can be delivered effectively into the lungs is limited. The therapeutic effect of a single treatment with Fab 19 was not sustained, so that a rebound in pulmonary virus titer occurred on the second day after treatment. However, this rebound in pulmonary RSV titer could be prevented by treating infected mice with a single dose of Fab 19 daily for 3 days. These observations suggest that human monoclonal Fabs produced in *Escherichia coli* may prove useful in the treatment of serious RSV disease as well as diseases caused by other viruses whose replication *in vivo* is limited primarily to the luminal lining of the respiratory tract. Passive prophylaxis will require whole IgG molecules derived from cloned Fabs because whole globulin has a considerably longer half life, *i.e.*, ~21 days, than Fab fragments.

## HEPATITIS VIRUSES SECTION

**Hepatitis E Virus (HEV).** The most recently recognized etiologic agent of human hepatitis is HEV. It is a small nonenveloped RNA virus, that based on electron microscopic appearance, most closely resembles members of the calicivirus family but, based on molecular analysis, is most closely related to rubella virus. It has a 7.6KB positive sense RNA genome that encodes three open reading frames. The nonstructural proteins are encoded at the 5' end of the





genome (ORF1), the putative capsid protein is encoded at the 3' end (ORF2) and a protein of unknown function is expressed from a small overlapping ORF (ORF3). The disease caused by this virus was first described 14 years ago by LID scientists. HEV is transmitted enterically and is endemic in developing countries of Asia and Africa. However, on occasion, it can cause large, explosive common source outbreaks when there is massive fecal contamination of the water supply. The epidemiology of HEV is similar to that of hepatitis A virus (HAV) but the latter is more readily transmitted and has a wider distribution worldwide. Hepatitis E is usually a mild, self-limited illness but in pregnant women it has a mortality rate of 20%.

Two important advances were made during 1993-94 that bring us closer to the control of hepatitis E. Cynomolgus monkeys were passively immunized with plasma from convalescent experimentally infected cynomolgus monkeys or were actively immunized with a recombinant protein encoded by the viral ORF-2 expressed by a baculovirus vector. Immunized and control animals were challenged with 1,000 to 10,000 cynomolgus ID<sub>50</sub> of the Pakistani strain of HEV. Each of the nonimmunized animals became viremic and developed abnormal liver histopathology. Passive immunization prevented or greatly decreased the abnormal liver histopathology and decreased the amount of virus in the feces but not in the blood. Actively immunized animals were completely protected against the development of abnormal liver histopathology. In addition, viremia and virus shedding in the feces were either prevented or greatly decreased. These observations identify the protein encoded by the viral ORF-2 as a major protective antigen and demonstrate the feasibility of immunization with recombinant HEV ORF-2 protein. Furthermore, the results of these studies clearly define the importance of serum HEV antibodies in resistance to infection. Although the virus can not be cultivated in cell culture at this time it should be possible to immunize at-risk individuals with recombinant HEV ORF-2 protein.

**Hepatitis A virus (HAV).** Hepatitis A virus (HAV) is a picornavirus with a single-stranded RNA genome of approximately 7500 nucleotides. Wild-type HAV grows poorly in cell-culture, generally is not cytopathic, and virus yields are low. A cell-culture adapted mutant has been selected which grows significantly more efficiently in cell-culture, is attenuated for marmosets and chimpanzees and has been licensed for use as an inactivated hepatitis A vaccine. Genetic analysis of these phenotypes continues to represent the central focus of LID's HAV research program.

In an effort to increase replicative capacity of the virus, viable chimeric viruses were constructed from two or more HAV strains including a virulent human strain, an attenuated strain, a vaccine strain, a cytopathic strain, and a simian strain. More efficient growth in cell culture translates to more efficient production of inactivated HAV vaccine which is currently licensed for use in Europe. The P2 region from a cytopathic strain of HAV was shown to confer the large plaque phenotype but not the lytic phenotype of the cytopathic virus. Viable HAV mutants under the translational control of the encephalomyocarditis virus internal ribosome site were also constructed. These chimeric viruses are being analyzed to determine if inclusion of this efficient control element enhances viral protein production and viral yield or affects host range.

The HAV/7 tissue culture-adapted mutant is attenuated in marmosets and chimpanzees. Because of the potential usefulness of this mutant in an attenuated live virus vaccine there was considerable interest in defining the genetic basis for its attenuation. Chimeric viruses were constructed for the purpose of defining its virulence genes. The 2A gene was identified as a second major HAV/7 determinant of attenuation for marmosets. Evidence was obtained that mutations in the 2A and 2C genes are almost totally responsible for the attenuation of the mutant. A full length infectious cDNA of the attenuated MRC-5 cell-adapted HAV vaccine strain derived from HAV/7 was constructed to serve as a genetic repository for the vaccine strain and to permit detailed molecular analysis of the virus it encodes. In addition, evidence was obtained that



mutations in the 5' non-coding region of the MRC-5 cell-adapted virus contribute to attenuation of the virus for marmosets.

**Hepatitis C Virus (HCV). Sequence Polymorphism in Core and Envelope Protein Genes.** Hepatitis C virus (HCV) is an important human pathogen that can cause acute and chronic hepatitis, cirrhosis and, possibly, hepatocellular carcinoma. This virus accounts for up to 25% of community-acquired hepatitis and over 90% of transfusion-associated hepatitis in the United States. The virus particles contain a positive polarity, single-stranded RNA genome with 5' and 3' noncoding (NC) regions. The core (C), envelope 1 (E1) and envelope 2 (E2) proteins are encoded at the 5' terminus and the nonstructural proteins are encoded at the 3' terminus of the single open reading frame of the genome. Furthermore, the finding of genetic heterogeneity of the HCV genome, especially in the genes encoding the envelope proteins, suggests that there may be heterogeneity similar to that seen in the envelope gene of human immunodeficiency viruses. Such a situation would bode ill for attempts at vaccine development. During 1993-94 our previous sequence analysis of the viral genome was extended to include the core gene, which is the most conserved HCV gene. The phylogenetic analysis of this gene was in agreement with that of the envelope 1 gene, which is highly variable. Additional analysis identified conserved features of the core gene that will be useful in understanding the role of the nucleocapsid protein in viral replication.

**HCV: Evidence That a Patient with Chronic HCV Infection Developed Neutralizing Antibodies to the Infecting Strain.** The development of an effective vaccine would be the most practical method for prevention of HCV infection, but it is not clear whether infection with HCV elicits protective immunity in the host. Neutralization of HCV *in vitro* was attempted with plasma of a chronically infected patient and the presence of residual infectivity was evaluated by inoculation of eight seronegative chimpanzees. The source of HCV was plasma obtained from a patient during the acute phase of post-transfusion hepatitis, which had previously been titered for infectivity in chimpanzees. Neutralization was achieved with plasma obtained from the same patient 2 years after the onset of primary infection, but not with plasma obtained 11 years later, although both plasmas contained antibodies against nonstructural and structural (including envelope) HCV proteins. Analysis of sequential viral isolates obtained from the same patient revealed a significant genetic divergence as early as 2 years after infection. However, the sequence of the HCV recovered from the patient 2 years after the infection had a striking similarity to that of the HCV recovered from one of the chimpanzees inoculated with the acute phase virus, suggesting that the progenitor of the new strain was already present 2 years earlier. This evidence, together with the sequence polymorphism of HCV recovered from the chimpanzees that received the same inoculum, confirms that HCV is present *in vivo* as a quasispecies. These observations provide the first experimental evidence from an *in vivo* study that HCV infection elicits a neutralizing antibody response in humans, but it appears that such antibodies are isolate-specific. This does not bode well for the successful development of a broadly reactive vaccine against HCV.

**Hepatitis B Virus (HBV): Analysis of Naturally Occurring HBV Surface Antigen Mutants and Continuing Elucidation of Genome Organization of HBV.** Hepatitis B virus (HBV) is the prototype of the hepadnavirus family, which includes hepatotropic viruses that infect at least six separate species. Infection with HBV causes polymorphic liver diseases including acute or fulminant hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Over 300 million people throughout the world are chronically infected with HBV and are at high risk of developing hepatocellular carcinoma, one of the world's most prevalent forms of cancer.





During 1993-94 two avenues of investigation were pursued. First, a putative neutralization escape mutant of HBV that emerged in individuals who were vaccinated against hepatitis B was studied to determine if the mutation that developed in the surface antigen (HBsAg) gene rendered the mutant resistant to immunity induced by current HBsAg subunit vaccines. Two licensed vaccines were investigated for their ability to protect chimpanzees against challenge with the HBs Ag mutant. Both recombinant vaccines provided protection against challenge with the mutant suggesting that individuals properly immunized with current vaccine are not at risk of infection by the mutant virus.

Second, molecular studies of the HBV genome revealed a bi-directional promoter complex within the X gene of the virus, further emphasizing the complex genetic organization of this virus. This information will be used to map new strategies for the control of hepatitis B.

**Woodchuck Hepatitis Virus (WHV): A Close Relative of HBV That is a Relevant Surrogate for Experimental Studies of HBV.** WHV is taxonomically and serologically related to HBV. Infection with each of these viruses is associated with acute and chronic hepatitis and hepatic cell carcinoma in their respective hosts and these associations appear to be etiological in nature. Thus, WHV infection of woodchucks provides a relevant and convenient model for understanding HBV infections of humans. Mutational analysis of the WHV X gene indicated that it is very sensitive to alterations and is essential for viral replication in animal transfection experiments. Fine mapping of the X transcript promoter was pursued to identify the essential nucleotides in this *cis*-acting element. For example, new insights were gained concerning the bi-directional promoter of WHV. Finally, two WHV isolates were shown to induce WHV antigenemia and liver tumors at significantly different rates. The latter finding has important implications for understanding the molecular mechanisms involved in viral replication and tumor development. Thus, it is now possible to design experiments to probe the mechanism of hepadnavirus oncogenesis.

**Search For New Hepatitis Viruses.** The objectives of this project are to identify and characterize previously unrecognized etiologic agents of hepatitis and to develop useful assays for diagnosis of infection and seroepidemiologic studies. A longer term objective is the development of passive and active immunoprophylaxis for these putative human pathogens. In 1989 a possible paramyxovirus etiology for giant cell hepatitis was proposed, based on electron micrographic changes seen in the livers of patients with this disease. Collaborative studies, including attempts to transmit the disease to primates, are in progress.

Patients with thalassemia in Sardinia, Italy, receive monthly blood transfusions as therapy. Many of these patients have developed chronic hepatitis. Most cases could be identified as hepatitis B or hepatitis C, but some lacked markers of either of these viruses. These patients are being studied for evidence of previously unrecognized hepatitis viruses by inoculation of appropriate specimens into experimental primates.

Patients with fulminant non-A, non-B hepatitis generally do not have evidence of infection with any recognized hepatitis virus. Only about 10% can be identified as having fulminant hepatitis C. The remaining 90% remain a diagnostic enigma and may be infected with one or more previously unrecognized viruses. In collaborative studies, attempts are underway to transmit the disease to primates.

Evidence for the existence of an additional water-borne hepatitis virus has come from seroepidemiologic studies of enterically transmitted non-A, non-B hepatitis in India. From 50-100% of hepatitis cases in 16 epidemics of water-borne hepatitis were caused by HEV but 1 water-borne epidemic was caused by neither HAV nor HEV.



## FLAVIVIRUSES (MOLECULAR VIRAL BIOLOGY SECTION)

**Dengue Viruses: Neurovirulence for Mice.** The flavivirus genus of the positive strand RNA *Flaviviridae* contains some 70 viruses most of which are arthropod-borne and cause disease of varying severity in humans and/or animals. The four serotypes of the dengue virus form a distinct antigenic subgroup. Dengue viruses continue to cause major epidemics in most tropical and subtropical regions where its *Aedes* mosquito vector is abundant. Infection by dengue virus usually causes fever, rash, and joint pain, but the disease is self-limited. However, in recent years, a more severe form of dengue, characterized by hemorrhagic fever and hemorrhagic shock, has occurred with an increasing frequency, most notably in the dengue epidemic region of southeast Asia. This form of dengue has a high fatality rate, especially in children and young adults.

Various strategies have been explored to produce an effective vaccine for prevention of dengue virus infection ever since the first dengue virus strain was isolated more than half a century ago. During early studies, serial intracerebral passage of dengue type 1 or type 2 virus in mice rapidly selected for mutants that were almost completely attenuated for humans. The rapid coordinate acquisition of mouse neurovirulence and attenuation for humans that occurs during a limited number of intracerebral passages in mice suggests that the genetic basis of these two phenotypes may be related. If this be so, then mouse neurovirulence might serve as a surrogate for attenuation. In this circumstance, identification of the genetic basis of mouse neurovirulence might concomitantly lead to the identification of mutations that attenuate dengue virus for humans.

In addition to the aforementioned mouse neurovirulent mutants of dengue type 1 and dengue type 2 virus, a similar neurovirulent mutant of dengue type 4 virus (DEN4) has been derived. The genetic basis for neurovirulence of the DEN4 mouse-adapted mutant was studied by comparing intratypic chimeric viruses that contained the three structural protein genes of the parental virus or its neurovirulent mutant on the background sequence of non-neurovirulent DEN4 strain 814669. The chimera that contained the three structural protein genes of the mouse neurovirulent mutant of DEN4 (strain H241) was highly neurovirulent in mice, whereas the chimera that contained the corresponding genes of its non-mouse adapted parent was not neurovirulent. Thus, some or all of the genetic loci for neurovirulence of the DEN4 mutant map within the structural protein genes which are located at the 5' end of the viral genome. Comparison of the structural proteins of the parent and its mouse neurovirulent mutant identified only five amino acid differences. Three of the mutations were located in the envelope (E) glycoprotein. When each of the E mutations was introduced separately into the parental virus sequence, two of the amino acid changes were identified as independent determinants of DEN4 mouse neurovirulence: (i) a single substitution of Ile for Thr (amino acid position 434 in the polyprotein or 155 in E) which ablated one of the two conserved glycosylation sites in DEN4 E yielded a virus that was almost as neurovirulent as the mouse-adapted mutant; and (ii) a Leu for Phe substitution (amino acid position 680 in the polyprotein or 401 in E) also yielded a neurovirulent virus that was less neurovirulent than the glycosylation mutant.

Similar studies were performed to elucidate the genetic basis for neurovirulence of the mutant derived 50 years ago by Sabin from the parental NGC strain of dengue type 2 virus. In this instance it also appears that some or all of the genetic loci responsible for neurovirulence are located in the structural protein genes. Sequence analysis of the cloned C-preM-E structural protein genes of parental and neurovirulent D2 NGC identified 7 mutations in the neurovirulent mutant which result in an amino acid change. Based on this information a series of chimeric viruses were constructed in which single or multiple mutations in pre-M and/or E were substituted into the parental sequence, and the resulting mutants were tested for neurovirulence



in mice. A mutation in the N-terminal one third of E protein which changed negatively charged glu (amino acid position 405 in the polyprotein or 126 in E) in the parental E protein to a positively charged lys appeared to be sufficient to produce the neurovirulence phenotype. Thus, acquisition of mouse neurovirulence by non-neurovirulent wild type dengue type 2 or type 4 requires only one to two strategic mutations in the envelope glycoprotein (E).

The mouse neurovirulent mutants of type 1 and type 2 viruses are also attenuated for humans. However, it is not clear that the neurovirulence mutations are sufficient for attenuating these dengue viruses for humans. Studies performed during 1993-4 suggest that restriction of replication of dengue virus in primate cell culture requires additional mutations. Analysis of neurovirulent DEN4 and its intratypic C-preM-E chimera indicate that the three mutations in E (amino acid positions 434, 435 and 680 in the polyprotein or 155, 156 and 401 in E) act collectively to prevent proper cleavage of preM to M; this event is required for virus maturation and acquisition of infectivity. In addition, the two mutations that are present in preM restrict growth in primate cells by a mechanism that does not involve an effect on cleavage of preM to M. It appears that attenuation may be more complex than neurovirulence but this issue requires further studies of this nature involving the type 1 and type 2 neurovirulent mutants that are demonstrably attenuated in humans. In contrast, the virulence of the type 4 mouse neurovirulent mutant for humans has not been evaluated.

**Dengue Viruses: Vaccine Development.** Success in developing methods for introducing site-specific mutations into the dengue type 4 virus (DEN4) positive strand RNA genome has provided a new strategy for the construction of safe and effective vaccines against dengue virus infection. A series of cDNA constructs was engineered to contain deletions ranging from 30 to 202 nucleotides in length in the 3' non-coding region of the genome. Full-length RNA transcripts of these DNA constructs were then tested for infectivity by transfecting permissive tissue culture cells. A panel of viable DEN4 mutants was recovered from mutant RNA transfected cells. Many deletion mutants were stable and produced plaques of reduced size on mosquito C6/36 cells compared to wild type virus. Analysis of the mutants in simian LLC-MK<sub>2</sub> cells revealed that most deletion mutants produced plaques that developed very slowly compared to wild type parental virus. Furthermore, most mutants that grew slowly in cell culture attained a lower titer than parental wild type virus. Growth-restricted deletion mutants were then evaluated for their infectivity and immunogenicity in rhesus monkeys and compared to the wild type parental virus. The infected monkeys exhibited fewer days of viremia compared to monkeys infected with the wild type virus. One deletion mutant induced an antibody response equivalent to that of wild type virus as measured by radioimmunoprecipitation and virus neutralization. In contrast, the other deletion mutants induced a low to moderate level of antibodies compared to parental virus. The immunogenicity of these 3' deletion mutants in monkeys appeared to correlate directly with their capacity to grow in simian LLC-MK<sub>2</sub> cells. Three deletion mutants of DEN4 which induced a moderate to high level antibody response in monkeys were selected for preparation of candidate vaccine seed lots. Initially, these virus suspensions will be evaluated in primates to confirm their attenuation and immunogenicity. The results of these studies in primates will determine whether clinical trials should be initiated and which mutants should be studied.

**Tick-Borne Encephalitis Virus Complex.** Previously, viable chimeric flaviviruses were constructed that contained the genes for tick-borne encephalitis virus (TBEV) structural proteins CME or ME with the remaining genes derived from dengue type 4 virus (DEN4). The ME chimera retained the neurovirulence for mice of its TBEV parent from which its M and E genes were derived, but it lacked the peripheral invasiveness of TBEV. The ME chimera was then subjected to mutational analysis in an attempt to reduce or ablate neurovirulence manifest when





virus is inoculated directly into the brain. Three distinct mutations were independently associated with marked reduction of mouse neurovirulence. These mutations ablated: (i) the TBEV PreM cleavage site which is required for proper processing of M protein; (ii) the TBEV E (envelope glycoprotein) glycosylation site; or (iii) the first DEN4 NS1 (non-structural protein one) glycosylation site. Each of the 3 attenuated mutants was restricted in growth in both simian and mosquito cells. Significantly, parenteral inoculation of mice with any one of these attenuated mutants induced complete resistance to fatal encephalitis during subsequent challenge with the highly neurovirulent ME chimera. These intriguing observations suggest a new strategy for developing live attenuated TBEV vaccines.

Unlike the highly virulent TBEV, the naturally occurring related Langat virus (LGT) is markedly less pathogenic for humans as well as mice. Genetic analysis of LGT may allow us to identify the molecular basis for neuroinvasiveness and neurovirulence of TBE viruses. The RNA genome of LGT (strain TP21) is 10940 nucleotides (nt) in length and contains an open reading frame for a polyprotein of 3,414 amino acids. The 5' noncoding region is 129 nt in length of which nts 1-25 and nts 80-128 are conserved in the corresponding regions of the related TBEV or Powassan virus (a TBE virus of North America). LGT contains 583 nt in the 3' noncoding region of which the last 90 nt are conserved among viruses of the TBE complex. It may be possible to develop a safe and effective live attenuated TBEV vaccine by constructing DEN4-LGT chimeric viruses that express the LGT protective antigens. Attenuating mutations similar to those identified earlier will be introduced in the chimeric virus genome and progeny virus will be analyzed for immunogenicity and complete loss of virulence.

## **GASTROENTERITIS VIRUSES (EPIDEMIOLOGY SECTION)**

### **Rotaviruses: Development and Evaluation of "Jennerian" Live Attenuated Vaccine.**

Rotaviruses are the single most important cause of severe diarrhea in infants and young children in both developed and developing countries, accounting for 30-50% of such illnesses. In developing countries, rotaviruses are a major cause of mortality during the first 2 years of life. Thus, the need for an effective vaccine is clear and as a consequence the rotaviruses have been assigned a top priority for accelerated vaccine development by the WHO.

Initially, we pursued the "Jennerian" approach to immunization. This strategy involves the use of an attenuated, antigenically-related live virus strain derived from a non-human host as the immunizing virus. Rhesus monkey rotavirus (RRV), which shares VP7 serotype specificity with human rotavirus type 3, was evaluated as the surrogate live virus vaccine strain in an extensive series of clinical trials. A bovine rotavirus has also been evaluated by us and other groups as well. This strategy has had limited success because oral administration of RRV or bovine rotavirus to infants less than six months of age did not consistently induce immunity against each of the four clinically important human rotavirus VP7 serotypes. We, therefore, developed a modified "Jennerian" approach that involved formulation of a quadrivalent rotavirus vaccine containing: (i) rhesus rotavirus (RRV) (VP7 serotype 3), and (ii) three human rotavirus-RRV reassortants, each possessing ten RRV genes and a single human rotavirus gene that encodes VP7 serotype 1, 2, or 4 specificity.

Thus far, a total of 8600 infants and young children in the United States or overseas have participated in placebo-controlled field trials of individual reassortant vaccines or the quadrivalent vaccine. Many of the studies have been completed, others are being analyzed, while a few are still in progress. The modified strategy developed in LID appears to be quite promising, especially with regard to preventing the most serious consequences of rotavirus infection, namely severe diarrhea. A significant reduction in the incidence of rotavirus diarrhea of any severity was



observed in the two most recent large clinical trials in the U.S. sponsored by Wyeth Laboratories. Protective efficacy against rotavirus diarrhea of any severity was 63% and 49%, respectively. Of particular importance was the observation that the quadrivalent LID vaccine was even more effective against severe rotavirus diarrhea, 80% protective efficacy. Significantly, the vaccine completely prevented the occurrence of rotavirus diarrhea severe enough to cause dehydration. These encouraging observations suggest that the LID live attenuated quadrivalent rotavirus vaccine will be licensed for wide spread use during the next few years. The vaccine is currently being developed as a pharmaceutical product by Wyeth Laboratories.

It is clear from our current understanding of the natural history of rotavirus infection that the goal of a rotavirus vaccine should be the prevention of severe dehydrating rotavirus diarrhea rather than the prevention of asymptomatic infection or mild illness. This is a realistic objective because under natural conditions prior rotavirus infection does not confer a high degree of protection against the subsequent occurrence of a mild rotavirus illness. In contrast, rotavirus infection usually induces solid resistance to subsequent severe rotavirus illness.

### **Rotaviruses: Development of Attenuated Human Rotaviruses by Cold-Adaptation.**

Group A rotaviruses possess two outer capsid proteins that function as independent neutralization antigens, namely VP4 (encoded by genome segment 4) and VP7 (encoded by genome segment 7, 8, or 9 depending on the strain). Although initially VP7 was thought to be the dominant neutralization antigen, recent studies have shown that VP4 is as effective as VP7 in inducing neutralizing antibodies during infection of experimental animals or susceptible infants or young children. Also, antibodies to VP4 or VP7 are independently associated with resistance of gnotobiotic piglets to experimental challenge with virulent rotavirus. However, VP7 is the only relevant rotavirus protective antigen present in candidate vaccines that are currently being evaluated for protective efficacy in humans. This is because these vaccines contain an animal rotavirus VP4 that is not related antigenically to the VP4 of any of the clinically important human rotaviruses.

In an attempt to maximize immunogenicity of live rotavirus vaccines by incorporating both protective antigens (*i.e.*, VP4 and VP7) of clinically important rotaviruses, LID scientists have pursued another approach to vaccine development in which cold-adapted (*ca*) and temperature-sensitive (*ts*) mutants of human rotaviruses were sought. This strategy entails the selection of such mutants during adaptation of virus to grow efficiently at suboptimal temperature.

Four antigenically distinct wild-type human rotaviruses of major epidemiological importance were subjected to passage in cell culture at suboptimal temperature in an attempt to isolate cold-adapted mutants that were attenuated for humans and suitable for use in a live viral vaccine. Mutants of each of these rotaviruses were selected during successive serial passage in primary African green monkey kidney cells at progressively lower suboptimal temperature (30°C, 28°C, and 26°C). The mutants exhibited both temperature sensitivity of plaque formation (*i.e.*, a *ts* phenotype) and ability to form plaques efficiently at suboptimal temperature (*i.e.*, a cold-adaptation [*ca*] phenotype) as compared to parental wild-type rotavirus. A detailed analysis of viruses recovered after the individual passages in the 30°C series indicated that the level of cold-adaptation observed at the end of the series was the result of two to three discrete incremental increases in ability to replicate and form plaques at 30°C. The succeeding set of ten serial passages at 28°C selected mutants that exhibited a greater degree of cold adaptation and mutants of three of the strains exhibited an associated increase in temperature sensitivity. Finally, in the case of three of the strains, the third successive serial passage series which was performed at 26°C selected for mutants with an even greater degree of cold adaptation that was associated with greater temperature sensitivity in one instance. This suggested that each of these viruses sustained a minimum of four to five mutations during the total selection procedure. If this be



the case, then such multiple mutations should enhance the genetic stability of the recovered *ca*, *ts* mutants. The mutations that were selected by passage at suboptimal temperature imposed a varying degree of growth restriction at physiologic temperatures (*i.e.*, 36°-39°C) *in vitro*. Recovery of mutants exhibiting a relatively wide range of growth restriction *in vitro* has provided a collection of viruses that can be searched for promising live vaccine candidate strains. The availability of such a disparate set of mutants should increase the likelihood of identifying promising vaccine mutants which exhibit the desired balance of attenuation and immunogenicity.

**Caliciviruses: Attempts to Cultivate Norwalk and Norwalk-Like Viruses in Cell Culture.** Caliciviruses, represented by the prototype Norwalk virus (NV) and Norwalk-like viruses, are the major cause of epidemic acute nonbacterial gastroenteritis in humans. A major obstacle to the study of these medically important viruses is our inability to grow these pathogens in cell culture. The molecular mechanisms responsible for the fastidious nature of these viruses are not known. The primary focus of a new project in LID is to gain a better understanding of the replication strategies of cultivatable strains of *Caliciviridae* and apply this information to the formulation of a successful strategy for the cell culture adaptation of the noncultivable viruses of this family. Feline calicivirus (FCV) was selected for analysis of its replication *in vitro* because it grows efficiently in cultured, permissive cells. Experiments were initiated with FCV to study receptor binding, infectivity of viral RNA, organization of the viral genome, protein processing, and the mechanisms responsible for host cell restriction in culture. Preliminary studies indicate that cell tropism of FCV involves functions subsequent to receptor binding, penetration and uncoating. Thus, FCV genomic RNA was infectious when transfected into a permissive cell line but infection did not occur when non-permissive cells were transfected.

Clones overlapping the entire Norwalk virus genome have been generated and recombinant proteins were successfully expressed *in vitro*. The functions of these proteins will be examined after the functions of FCV proteins have been elucidated. In addition, we will continue to search for a cell strain or line or possibly an organ culture system in which Norwalk virus will replicate.

**Caliciviruses: Antigenic Diversity and Epidemiology.** Although the Norwalk and Norwalk-like human caliciviruses are major etiologic agents of epidemic nonbacterial gastroenteritis, knowledge of the molecular epidemiology of these noncultivable viruses is limited. In 1990, the genome of Norwalk virus (NV) was cloned and sequenced by Jiang and Estes. Since that time, LID scientists have obtained sequence information for several other Norwalk-like viruses, including the entire capsid protein gene (corresponding to ORF2 of the viral genome) of the Desert Shield virus (DS395), the Toronto virus (TV24) and the Hawaii virus (HV). The latter is an important Norwalk-like virus reference strain that was previously identified in LID as being serotypically distinct from Norwalk virus. The uniqueness of Hawaii virus (HV) was established during cross-challenge studies in volunteers and also by immune electron microscopy (IEM).

Comparison of the amino acid sequence identities of the capsid protein of HV, NV and three other human caliciviruses (TV24, DSV395, and Southampton virus [SHV]) identified two major genetic groups (genogroups) represented by HV and NV. Hawaii virus showed 76% amino acid identity with TV24, and 48% identity with NV, DSV395, or SHV. In addition, comparison of part of the viral polymerase gene of HV with that of other human caliciviruses confirmed the distribution of strains into two genogroups represented by HV and NV.

Consensus sequence analysis of the capsid protein of HV (546 amino acids), DSV395 (544 aa), NV (531 aa), SHV (546 aa), and TV (548 aa) indicated that most of the conserved sequence was located in the amino terminal half (amino acids 1-218) and the carboxy terminal





region (amino acids 416-544). The conserved regions flank a region of sequence variability which probably encodes a major antigenic site.

The capsid protein of HV, DSV395, TV24 and NV was expressed in a rabbit reticulocyte lysate translation system yielding a 58-60 kD protein which is consistent in size with the capsid protein of animal caliciviruses. Shared antigenic sites were identified when the genetically diverse human calicivirus capsid proteins were analyzed by immunoprecipitation using homologous and heterologous paired sera from infected individuals.

Self-assembled virus-like particles (VLPs) similar in dimensions to calicivirus virions were produced when the capsid protein gene of various Norwalk-like viruses was expressed by a baculovirus vector. Immune electron microscopy (IEM) has been the "gold standard" for establishing serologic relationships among the noncultivable human caliciviruses. For this reason, IEM experiments were performed with recombinant Desert Shield (rDSV) and rNV VLPs in an attempt to define their antigenic relatedness by this established method. Desert Shield virus (DSV) and NV VLPs were distinct when rabbit hyperimmune antisera prepared against VLPs and paired human sera were tested by IEM. This indicated that members of the same genogroup can exhibit serotypic diversity. These VLPs might prove useful in immunoprophylaxis if a need for immunization is identified during future epidemiological studies.

## **SIMIAN IMMUNODEFICIENCY VIRUSES (SIV) (IMMUNODEFICIENCY VIRUSES SECTION)**

**SIV: Pathogenesis.** A crucial element in the development of effective therapeutic and prophylactic strategies for AIDS is an experimental animal model in which infection produces effects that parallel the pathogenesis of the human disease. SIV infection of macaques appears to be such a model because this virus induces an immunodeficiency syndrome in infected macaques that is remarkably similar to human AIDS. In addition, SIV utilizes the CD4 molecule as a receptor, and the pathogenesis of disease appears similar. An important use of this animal model system is the detailed study of pathogenesis and viral determinants of disease since such prospective analysis is often not feasible in humans. Studies of this type will allow us to determine how these primate lentiviruses destroy the immune system of their host, and this understanding should prove useful in the development of more rational therapeutic antiviral strategies.

A continuing major effort in the Section has been the study of SIV pathogenesis in experimentally- infected macaques, utilizing strains derived from sooty mangabeys (SIVsm) and African green monkeys (SIVagm). SIVsm clones derived from an immunodeficient macaque were employed to study viral factors contributing to the induction of AIDS. A study of SIVsm clones derived from the spleen of an immunodeficient SIV-infected pig-tailed macaque identified a region analogous to the V3 loop of HIV-1 envelope to be responsible for determining tropism. Naturally-occurring variation in this region determined whether virus infected macaque lymphocytes and/or macrophages and this tropism appeared to be linked to pathogenicity. The degree of variation of the envelope glycoprotein (env) of viruses within tissues of SIV-infected macaques was compared utilizing single stranded polymorphism (SSCP) and sequence analysis. Virus populations within lymphoid tissues were highly heterogeneous whereas, distinct and homogeneous populations were present in the brain and peripheral blood mononuclear cells (PBMC).

A SIVagm strain (SIVagm9063) was isolated from an African green monkey (AGM) and molecularly cloned after experimental passage through a pig-tailed (PT) macaque. This isolate induced immunodeficiency in experimentally infected pig-tailed macaques but caused an asymptomatic infection in rhesus macaques and African green monkeys. Pig-tailed macaques



infected with the SIVagm molecular clone developed a high viral load and subsequently progressed to develop the immunodeficiency syndrome. In contrast, pig-tailed and rhesus macaques failed to develop disease. Analysis of tissues from infected African green monkeys, rhesus monkeys, and pig-tailed macaques indicated that virus replication and dissemination were markedly restricted in the former two simian species compared to pig-tailed macaques. This suggested that viral load was directly related to the development of disease.

Because lymphocyte activation plays such an important role in lentivirus replication and because of the importance of lymphokines and cytokines in this process, we tested the effect of an inhibitor of TNF  $\alpha$  (pentoxifylline) on SIV infection of macaques. Treatment of infected monkeys failed to alter the course of infection or disease.

**SIV: Vaccine Development.** The SIV/macaque experimental model of AIDS has also been used to evaluate various vaccine strategies. The ability of inactivated whole SIV vaccine to modify the subsequent course of disease was assessed in a group of five immunized macaques challenged with cell-associated SIV. As predicted from previous experiments, each of the macaques became infected; however, long-term surveillance revealed that the immunized animals survived longer than the naive controls suggesting that this form of immunization might beneficially modify the course of disease.

The highly attenuated "modified vaccinia virus of Ankara" (MVA) was used to construct recombinants that expressed the SIVsm gag-pol and env. MVA was chosen for this purpose because it is highly attenuated having sustained deletion of 15% of its viral genome during 600 serial passages in chick embryo cell culture. The MVA expresses foreign genes efficiently but the virus does not spread to contiguous cells because infectivity is blocked at the late stage of virus assembly. The immunogenicity and protective efficacy of the MVA recombinant (MVA-SIV) was compared with that of a similar recombinant prepared from the fully competent Wyeth vaccinia virus (Wyeth-SIV). Each recombinant virus was used to immunize 4 macaques a total of five times at approximately 3 month intervals. Whereas, the MVA-SIV boosted SIV antibody levels following sequential inoculations, the Wyeth-SIV recombinant only induced a boost after the second immunization and antibody levels declined subsequently. Thus, MVA-SIV appeared to more immunogenic than Wyeth-SIV. Although immunization did not prevent infection following intravenous challenge with uncloned SIVsm, the virus load of three of the four MVA-SIV-immunized animals was 100- to 1,000-fold reduced compared to animals immunized with control nonrecombinant vaccinia virus. Also, plasma viremia was not detected in the MVA-SIV group. Thus, immunization with MVA-SIV resulted in significant restriction of virus replication and dissemination which may be predictive of longterm survival of these animals.

Studies with MVA-SIV recombinants will be expanded because of the promising results of the initial trial. Future studies will focus upon: (i) improving the degree of protection; (ii) determining the SIV proteins and immune responses responsible for the observed protective effect; and (iii) longterm clinical follow-up of immunized animals. A vaccine trial to confirm the reduction in virus load in MVA-immunized animals has been initiated. The immunization regimen will be reduced to a schedule of three inoculations and the efficacy of MVA-SIV as the sole immunogen will be compared with MVA-SIV boosted with inactivated whole SIV. These animals will be challenged with uncloned SIVsmE660 and virologic and immunologic events will be monitored during long-term post-challenge follow-up.

We will attempt to improve the immunogenicity of the final booster inoculation by using a secreted oligomerized SIV env constructed by removing the cleavage site between gp120 and gp40 and deleting the transmembrane spanning domain. This envelope protein will be expressed in vaccinia virus and partially purified by lectin affinity. The SIV proteins involved in protection will be determined by generating and testing MVA recombinants expressing env or gag-pol individually. Accessory proteins such as Nef, Vif, Vpr, Tat, and Rev will be expressed in MVA





to determine whether these proteins are targets of cell mediated immune response. Finally, the ability of these MVA-recombinants to generate a cytotoxic T cell response will be determined in genetically defined rhesus macaques in collaboration with Dr, Norman Letvin (Beth Israel Hospital, Harvard Medical School).



## HONORS AND AWARDS

### **Robert M. Chanock, M.D.**

Recipient of 1993 Presidential Meritorious Executive Rank Award.

Recipient of Bristol-Myers Squibb Third Annual Award for Distinguished Achievement in Infectious Disease Research, December 7, 1993.

Invited to be the Second Annual Frederick C. Robbins Lecturer, Case Western Reserve University, Cleveland, Ohio, December 2, 1993.

Invited to present lecture, "Respiratory syncytial virus and modern vaccination strategies" at the Dedication of the Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden, May 5-6, 1994.

Member of National Academy of Sciences Committee on International Security and Arms Control, Working Group on Biological Weapons Control. Participated in Bilateral Meetings with members of Russian Academy of Sciences, Moscow, April 18-21, 1994, and Scientific Aspects of International Security Group, Royal Society, London, April 22, 1994.

Co-Organizer of Symposium on Modern Approaches to New Vaccines Including the Prevention of AIDS, Cold Spring Harbor Laboratory, Long Island, New York, October, 1994.

### **Vanessa M. Hirsch, D.V.M., D.Sci.**

Chairperson and invited speaker, Workshop on HIV/SIV Pathogenesis and Mucosal Transmission, Bethesda, Maryland, March, 1994.

Chairperson, Retroviruses 1994, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, May, 1994.

Presentation to the Discovery Subcommittee to the National Task Force on AIDS Drug Development, July, 1994.

Chairperson, organizer and invited speaker at the 12th Symposium on Nonhuman Primate Models for AIDS, Boston, Massachusetts, October, 1994.

### **Albert Z. Kapikian, M.D.**

Invited speaker at Workshop on "Trends in Vaccine Development" at the 9th International Congress of Virology, Glasgow, Scotland, August 8-13, 1993. Presentation entitled "Efficacy of the 'Jennerian' and modified 'Jennerian' approach to vaccination against severe rotavirus diarrhea," August 11, 1993.



Invited to deliver lecture on Viral Gastroenteritis at FAES Graduate School Virology Course, January 12, 1994.

Invited to attend (as non-member) Advisory Committee on Immunization Practices Meeting at CDC, Atlanta, Georgia, June 30, 1994.

### **Ching-Juh Lai, Ph.D.**

Invited to present lecture, "Development of a Molecular Vaccine Strategy Against Dengue Infections," April 8, 1994, at The University of Texas Medical Branch at Galveston, Houston, Texas.

Invited to present lecture, "Development of a Molecular Vaccine Strategy Against Dengue Infections," at the National Taiwan University College of Medicine, Taipei, Taiwan, May 23-28, 1994.

### **Brian R. Murphy, M.D.**

Organizer of WHO Meeting on Development of Vaccines Against Diseases Caused by RSV and PIV3, Nyon, Switzerland, March 27, 1994.

Invited to present lecture at the American Association of Immunologists/SMI symposium "Mucosal Vaccines: Recent Developments in Design and Human Analysis," April 24-28, 1994, in Anaheim, California. Lecture entitled "Mucosal immunity to respiratory viral infections."

Invited to present the Bageley Oration entitled "Immunization against respiratory viruses" at an Australian Society for Microbiology Annual Meeting in September, 1994.

Invited to present lecture entitled, "Respiratory viruses--vaccine development," in Symposium entitled "Respiratory Viruses: Epidemiology and Control," at the Australian Society for Microbiology Annual Meeting, September, 1994.

Invited speaker, St. Jude's Children's Hospital Virology Seminar Series, "Immunobiology of RSV," October, 1993.

### **Robert H. Purcell, M.D.**

Invited to present lecture, "Recent HCV Infectivity Studies" at Abbott Laboratories, November 1, 1993.

Invited to present lecture, "Viral Safety - Hepatitis A," March 16, 1994, at the Seventh Immuno Study Update, St. Thomas, U.S. Virgin Islands, March 14-16, 1994.





**Peter L. Collins, Ph.D.**

Invited to present lecture, "Molecular studies of non-segmented negative strand RNA viruses using biologically-active cDNA-encoded analogues of genomic RNA," January 7, 1994, at the Virus Group Symposium, University of Warwick, London, United Kingdom, January 5-7, 1994.

Invited to present lecture "Molecular studies of non-segmented negative strand RNA viruses using biologically-active cDNA-encoded analogues of genomic RNA," at World Health Organization meeting, March 27, 1994.

**James E. Crowe, Jr., M.D.**

Invited to participate in Grand Rounds at Minneapolis Children's Medical Center, November 20, 1993. Title "Immunoprophylaxis of RSV Disease."

Invited to present lecture "Live attenuated RSV vaccines for respiratory syncytial virus," at World Health Organization meeting, March 27, 1994.

**Suzanne U. Emerson, Ph.D.**

Invited to present seminar on Hepatitis A Virus at Uniformed Services University of the Health Sciences, November, 1993.

Invited speaker on Hepatitis E Virus for Medical Virology Club at American Society of Virology Meeting, Madison, Wisconsin, July 9, 1994.

**Susan M. Hall, Ph.D.**

Invited to present lecture "Vaccines for PIV3" at Aviron, December 20, 1993.

Invited to present lecture "Progress toward the rescue of a full-length clone of PIV3" at World Health Organization meeting, March 27, 1994.

**Eileen N. Ostlund, D.V.M., Ph.D.**

Invited to present lecture, "Identification of distinct equine rotavirus VP4 serotypes and cold adaptation of equine rotavirus H2" at the Seventh International Congress of Equine Infectious Diseases, Tokyo, Japan, June 8-11, 1994.



## PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Parainfluenza Virus Type 3 (PIV3) Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI: Peter L. Collins, Ph.D.

Tenured Scientist

LID, NIAID

Others: Kenneth D. Dimock, Ph.D.

N.I.H. Special Volunteer

LID, NIAID

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Infectious Diseases

## SECTION

Respiratory Viruses Section

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

## TOTAL STAFF YEARS:

0.75

## PROFESSIONAL:

0.25

## OTHER:

0.50

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previously, we developed an experimental "rescue" system for human parainfluenza virus type 3 (PIV3) based on a cDNA-encoded minigenome, PIV3-CAT. This is a truncated version of PIV3 genome RNA in which all of the viral genes have been removed and replaced by the bacterial chloramphenicol acetyl transferase (CAT) gene. When synthesized *in vitro* and transfected into cells which have been infected with standard PIV3 helper virus, PIV3-CAT was rendered biologically active ("rescued") as evidenced by the intracellular expression of CAT and by the production of a component (presumably infectious particles) that was released into the medium and could be passed productively onto fresh cells in the presence of helper virus. Together with a similar system developed by us for human respiratory syncytial virus (RSV) (accompanying report) and by others for Sendai virus, this represents the first experimental method by which one can synthesize, and thereby manipulate, biologically-active analogs of the genome of a nonsegmented negative strand virus. It will now be possible to directly identify and characterize *cis*-acting signals in genome and, as described here, considerable progress has been made in this area. The success in rescuing PIV3-CAT supports the idea that it will be possible to adapt the system such that a complete synthetic genome can be used to produce nondefective virus. This would make possible the creation of viruses that contain defined mutations for vaccine and experimental purposes.





## PROJECT DESCRIPTION

PIV3, an enveloped RNA-containing paramyxovirus, is second only to RSV as a major etiologic agent of pediatric viral respiratory tract disease. PIV3 genome is a single negative-sense strand of 15,463 nt. Previously, we identified and mapped the six major viral mRNAs encoded by the PIV3 genome, determined the structures of the intergenic regions in genome RNA, and cloned and sequenced five of the six major viral mRNAs. PIV3 was shown to encode six major structural proteins: the hemagglutinin-neuraminidase (HN) attachment glycoprotein (69-70 kDa), fusion (F) glycoprotein (60-63 kDa), nonglycosylated inner membrane (M) protein (35-40 kDa), large (L) polymerase protein (200 kDa), major nucleocapsid protein (NP, 66-68 kDa), and phosphoprotein (P, 83 kDa). A second open reading frame (orf) in the P mRNA encodes the nonstructural (C) protein (22 kDa), and there may be one or two additional small proteins (D and V) encoded by other orfs in the P mRNA.

Recently, we developed a new genetic system, namely the rescue system for minigenomes, which will allow experimental manipulations that were not previously possible for any of the nonsegmented negative strand viruses. We have been using this system to characterize cis-acting signals in the PIV3 genome

## MAJOR FINDINGS

We previously constructed cDNA that encodes PIV3-CAT, a negative-sense, truncated version of PIV3 genome that contains the CAT reporter gene in place of the viral genes. Thus, the encoded PIV3-CAT RNA contains, in 3' to 5' order: the 3' terminal 55-nt extragenic leader region, the adjoining 10-nt NP gene-start signal (a conserved sequence motif found at the start of each gene), the 3' 46-nt nontranslated region of the NP gene, the 670 nt CAT orf, the 5' 58-nt noncoding region of the L gene, the 13-nt L gene-end signal (a conserved sequence motif found at the end of each gene), and the 44-nt 5'-terminal extragenic trailer region. Transcription *in vitro* of HgaI-linearized cDNA yields a 896-nt negative sense genome analog containing the correct ends. In a second construct, the HgaI site was replaced with DNA encoding the hepatitis delta virus self-cleaving ribozyme to generate RNA with the correct 3' end.

The transfection of PIV3-CAT genome into PIV3-infected cells resulted in the expression of CAT. The PIV3 helper was strictly required. Activity was undiminished by treatment of the transfecting nucleic acid with DNase or by treatment of the cells with actinomycin D, consistent with the idea that expression was driven by the PIV3 polymerase acting on RNA. Passage of the supernatant fluids onto fresh cells 24 h later resulted in the expression of CAT, indicating that the synthetic genome was incorporated into some type of transmissible form which resembled a virus particle in being sensitive to neutralizing antibody directed against the PIV3 HN protein.

A second version of PIV3-CAT DNA was constructed to encode PIV3-CAT RNA as a positive-sense strand, which would correspond to the predicted replicative intermediate. The positive-sense version of PIV3-CAT was approximately one-fifth as active in the rescue assay as was the negative-sense version. The negative-sense version was used in the experiments described below.

We also constructed two versions of a dicistronic minigenome which contains the CAT and luciferase (luc) genes under the control of independent gene-start and gene-end signals and separated by an intergenic triplet. In one version, PIV3-CAT-luc, the CAT gene is first in the gene order, whereas in the second version, PIV3-luc-CAT, it is second. The two versions



expressed CAT with equal efficiency, and analysis of luciferase expression remains to be done. An analogous dicistronic RSV-CAT-luc construct has been shown to express each gene as a separate subgenomic mRNA which is efficiently transcribed into protein (accompanying report).

The efficiency of CAT expression increased 3,000-fold during nine serial passages, suggesting that PIV3-CAT was replicated more efficiently than the helper virus. The structure of PIV3-CAT genome resembles that of one variety of defective interfering (DI) particles common to paramyxoviruses. In its current form, however, PIV3-CAT did not detectably interfere with helper virus replication as determined by plaque assay. We are now investigating whether further modifications to PIV3-CAT might increase its replication efficiency such that it can interfere with standard PIV3. One such modification will be to eliminate its capacity to transcribe, based on the supposition that this would result in a concomitant increase in the synthesis of replicative intermediate. A second modification will be to replace the 3' end of PIV3-CAT with the sequence from the 3' end of replicative intermediate RNA, based on the supposition that this sequence might be a more powerful promoter.

Studies to date have provided the following observations regarding the *cis*-acting sequences in the PIV3 minigenome:

**Mapping *cis*-acting sequences.** Insertion of a foreign di- or hexanucleotide into any of the following regions ablated rescue: (i) the entire leader region, (ii) the gene-start or gene-end signals, (iii) the leader-proximal half of the nontranslated NP gene sequence which precedes the start of the CAT orf, and (iv) the last ~10 nucleotides of the trailer region. We interpret this as follows. The gene-start and gene-end signals probably are requisite transcription signals. Inactivation of either signal would interfere with the synthesis of the predicted subgenomic CAT mRNA, thus ablating CAT expression. However, these signals probably are not important for replication, a point which will have to be addressed by direct analysis of intracellular PIV3-CAT RNA. The sequence at the end of the trailer region probably encodes the promoter in replicative intermediate RNA, and thus probably is a critical replication signal. Finally, the observed sensitivity of the entire leader region and much of the adjoining NP noncoding region is intriguing. A likely possibility is that these regions contain both replication and transcription signals. There might be a replication signal (promoter) at the first ~10 nucleotides of the leader region corresponding to the one proposed to be at the 3' end of replicative intermediate RNA. This might be overlapped by a transcription signal which extends into the NP noncoding region. Thus, the distribution of *cis*-acting signals in PIV3 appeared to be different than for RSV (accompanying report), in which the leader region contained two circumscribed signals and the nontranslated region of the first gene was not essential.

**Saturation mutagenesis of the 3' promoter region.** The first 12 nt at the 3' end of PIV3-CAT RNA were characterized by "saturation" mutagenesis, in which each position is changed in turn to each nt alternative. This showed that the first position at the 3' end was very sensitive to substitution, that positions 3-8 were moderately-to-highly sensitive to most substitutions, and that positions 10-12 were unaffected by substitution. The length of this putative promoter was similar to that defined for RSV by saturation mutagenesis, and common features of both sequences include the presence of a U at the 3' end and a high content of U (7 of the first 12 nt). Otherwise, the patterns of sequence sensitivity for PIV3 and RSV bore little resemblance to each other, perhaps reflecting the extensive evolutionary divergence between the two.

Whereas RSV-CAT could accommodate the addition of heterologous extensions of up to eight nucleotides to its 3'-end, the addition of even a single nucleotide to the 3' end of PIV3-CAT was strongly inhibitory.



**Characterization of *cis*-acting sequences at gene junctions.** The dicistronic PIV3-CAT-luc and PIV3-luc-CAT constructs are being used to map and characterize the functions of the sequence motifs located at the intergenic boundaries. The various naturally-occurring gene junctions were compared in this system and found to be approximately equivalent, suggesting that these naturally-occurring differences do not have major functional consequences.

Deletion of the intergenic region ablated expression of the downstream gene, providing the first evidence that this is a critical signal. Thus, the PIV3 intergenic triplet might be a functional part of the downstream gene-start signal. This differs from the situation with RSV, where the intergenic regions can be ablated with minimal effect. PIV3 and RSV illustrate the two types of intergenic structures found among paramyxoviruses, namely conserved versus nonconserved, and these results show that these structural differences are of functional significance.

Deletion of the gene-end signal of the upstream gene greatly reduced its expression and ablated expression of the downstream gene. It seems likely that, in the absence of this signal, the polymerase continues transcription so as to make a dicistronic mRNA. This would preclude the synthesis of a separate mRNA from the downstream gene. The reduction in expression of the upstream gene at the protein level is not understood, although presumably the dicistronic mRNA is translated inefficiently for some reason.

The length and sequence of the intergenic region seemed to be important, but some variation was permitted. Insertion of a second intergenic triplet was lethal, but replacement of the triplet with certain dinucleotides of related sequence was only moderately inhibitory. In addition, certain point substitutions were tolerated.

Intriguingly, the two mutations which ablated expression of the downstream gene alone, namely deletion of the intergenic region or the gene-start sequence of the downstream gene, had the unexpected effect of increasing the expression of the upstream gene by up to 10-fold. This might be explained by postulating that these signals impede the progression of the polymerase down the genome, and that their removal would allow the passage of many more polymerases resulting in a higher rate of transcription. This model also was suggested by the previous finding that, for the distantly-related rhabdovirus vesicular stomatitis virus, the polymerase indeed does pause for relatively long periods of time at the intergenic junctions during *in vitro* transcription.

Another reason for developing the PIV3-CAT system was that it can be used to optimize methods for rescuing complete, nondefective synthetic PIV3 genome encoded by cDNA. This project is under development by others in the LID. The success in rescuing PIV3-CAT demonstrates that cDNA-encoded genome indeed can be rendered biologically active. The finding that replicative intermediate RNA can be rescued suggests that this might be the optimal way of introducing a full-length RNA into cells for rescue: a naked negative-sense copy might be inactivated by hybridization to viral mRNAs before it can be encapsidated. The PIV3-CAT system also will be useful to map and evaluate attenuating mutations in PIV3 live vaccine strains which are under evaluation as potential live vaccines.

## PUBLICATION

Dimock K, Collins PL. Rescue of synthetic analogs of genomic RNA and replicative-intermediate RNA of human parainfluenza virus type 3. *J Virol* 1993;67:2772-2778.





## **PROJECT DESCRIPTION IN LAY LANGUAGE (PDLI)**

**Laboratory:** Laboratory of Infectious Diseases  
**Principal Investigator:** Peter L. Collins, Ph.D.  
**Z01 Number:** Z01-AI 00323-13 LID

**SCIENTIFIC AREAS OF INTEREST (SAIs):**  
63, 68, 69

### **SUMMARY OF WORK IN LAY LANGUAGE:**

We developed a new, simple system for the direct manipulation of sequences of human parainfluenza virus type 3 (PIV3) genome RNA. DNA was constructed to encode a truncated version of genome RNA in which all of the viral genes have been removed and replaced by an enzyme marker gene (bacterial chloramphenicol acetyl transferase [CAT]) whose expression could be easily monitored. When introduced into tissue culture cells which had been infected with standard PIV3 to supply viral proteins, the synthetic minigenome RNA was rendered biologically active ("rescued") as evidenced by the intracellular expression of CAT and by incorporation of the PIV3-CAT minigenome RNA into infectious particles. Together with a similar system developed by us for human respiratory syncytial virus (RSV) (accompanying report) and by others for Sendai virus of mice, this represents the first experimental method for manipulating genome sequences of a nonsegmented negative strand virus (a large group of viruses which includes rabies, mumps, measles, canine distemper, rinderpest and numerous other medically- or economically-important pathogens). It will now be possible to directly identify and characterize the roles of genome sequences and viral proteins in replication, transcription and virion morphogenesis. The success in rescuing the PIV3-CAT minigenome is an important step in our work to develop methods by which complete infectious PIV3 can be made from synthetic RNA prepared from cloned DNAs. This capability would allow us to produce attenuated vaccine strains and to make viral mutants for the purpose of elucidating the elements important in viral pathogenicity and disease.

### **IMPLICATIONS OF RESEARCH:**

PIV3 is second only to RSV as an important, ubiquitous viral agent of serious respiratory tract disease in humans, especially in infants and children. This work also would be relevant to related viruses which have negative-sense RNA genomes, including mumps, measles, canine distemper, rinderpest, Sendai, vesicular stomatitis, rabies, Ebola, and the constellation of other parainfluenza viruses of humans and animals.

This experimental system (together with a similar one developed by us for RSV [accompanying report] and by others for Sendai virus) provides the first method for introducing a synthetic genome-like RNA of this particular type of virus (nonsegmented negative strand virus) into the viral replicative cycle. We have used the system to identify and characterize sequences in genome RNA that are critical for RNA replication and viral gene expression. It is expected that this line of work will lead to the development of methods for producing complete infectious virus from synthetic RNA produced from cloned DNA. Mutants made in this way would be invaluable for basic studies designed to characterize virus replication and pathogenicity. Pertinent to vaccine development, this would make it possible: (i) to directly identify attenuating



mutations in existing viruses, (ii) to create new types of attenuating mutations, (iii) to combine constellations of attenuating mutations to create a graded series of vaccine viruses, and (iv) to maintain a stable seed for vaccine strains. It also is conceivable that this type of rescue system would have applications in gene therapy of the respiratory tract.

#### **RECENT ACHIEVEMENTS/DEVELOPMENTS:**

We have been able to introduce minigenomes into the PIV3 replicative cycle. This is the first step in developing methods for producing PIV3 from synthetic RNA prepared from cloned DNA, which has not been achieved for any of the nonsegmented negative strand RNA viruses. Using the minigenome system, we have mapped most of the critical sequences in the genome and have made new findings regarding the mechanism of gene transcription.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00323-13 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Parainfluenza Virus Type 3 (PIV3) Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Peter L. Collins, Ph.D. Tenured Scientist LID, NIAID

Others: Kenneth D. Dimock, Ph.D. N.I.H. Special Volunteer LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Respiratory Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.75

PROFESSIONAL:

0.25

OTHER:

0.50

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previously, we developed an experimental "rescue" system for human parainfluenza virus type 3 (PIV3) based on a cDNA-encoded minigenome, PIV3-CAT. This is a truncated version of PIV3 genome RNA in which all of the viral genes have been removed and replaced by the bacterial chloramphenicol acetyl transferase (CAT) gene. When synthesized *in vitro* and transfected into cells which have been infected with standard PIV3 helper virus, PIV3-CAT was rendered biologically active ("rescued") as evidenced by the intracellular expression of CAT and by the production of a component (presumably infectious particles) that was released into the medium and could be passed productively onto fresh cells in the presence of helper virus. Together with a similar system developed by us for human respiratory syncytial virus (RSV) (accompanying report) and by others for Sendai virus, this represents the first experimental method by which one can synthesize, and thereby manipulate, biologically-active analogs of the genome of a nonsegmented negative strand virus. It will now be possible to directly identify and characterize *cis*-acting signals in genome and, as described here, considerable progress has been made in this area. The success in rescuing PIV3-CAT supports the idea that it will be possible to adapt the system such that a complete synthetic genome can be used to produce nondefective virus. This would make possible the creation of viruses that contain defined mutations for vaccine and experimental purposes.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00324-13 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Laboratory Studies of Influenza Viruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Brian R. Murphy, M.D.	Head, RV Section	LID, NIAID
Others:	Robert M. Chanock, M.D.	Chief	LID, NIAID
	E. Kanta Subbarao, M.D.	Senior Staff Fellow	LID, NIAID
	Cassandra Lawson, Ph.D.	Visiting Associate	LID, NIAID
	Eun Ju Park, Ph.D.	Visiting Fellow	LID, NIAID

COOPERATING UNITS (if any)

FDA (Epstein); LVD/NIAID/NIH (Bennink, Yewdell); University of Rochester (Treanor)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Respiratory Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

4.5

PROFESSIONAL:

2.0

OTHER:

2.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Last year a new strategy was developed for the construction of a live attenuated influenza A virus vaccine in which attenuating mutations were introduced into a cDNA copy of the PB2 gene by site-directed mutagenesis and the mutant PB2 gene was subsequently rescued into an infectious virus. This year we have extended these initial findings considerably by demonstrating the feasibility of introducing two *ts* mutations into the PB2 gene that specify a greater degree of attenuation and temperature sensitivity than that specified by single a *ts* mutation. A double mutant was constructed that had *ts* mutations at amino acid positions 265 and 556 in the PB2 gene of the A/AA/6/60 virus. This double mutant was more temperature sensitive and more attenuated than a transfectant bearing a single mutation at position 265 or 556. Thus, in the near future it should be possible to construct a PB2 gene that specifies an increased level of attenuation that is very stable. Such a PB2 gene with multiple mutations could be used alone, or in conjunction with another attenuating gene, to attenuate new epidemic influenza A viruses as they emerge in nature.

A mutation in the PA polymerase protein at amino acid residue 245 or 347 can suppress the *ts* and attenuation phenotypes specified by an asn-ser mutation at position 265 in the PB2 gene.

Primary pulmonary cytotoxic CD8+ T-cells induced by immunization of mice with vaccinia recombinants expressing the major influenza A virus NP CTL epitope did not protect the mice against challenge with virulent virus.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00325-13 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of Respiratory Viruses in Primates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Brian R. Murphy, M.D.	Head, RV Section	LID, NIAID
Others:	James E. Crowe, M.D.	Clinical Associate (CO)	LID, NIAID
	Robert M. Chanock, M.D.	Chief	LID, NIAID
	Jinlin Du, M.D.	Visiting Fellow	LID, NIAID

COOPERATING UNITS (if any)

BIOQUAL, Inc., Rockville (Bradbury); Wyeth-Ayerst Research (Lubeck)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Respiratory Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.25

PROFESSIONAL:

1.25

OTHER:

1.00

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A major goal of the laboratory is the development of a live attenuated bivalent respiratory syncytial virus (RSV) vaccine. The LID/NIAID has entered into a CRADA with Wyeth-Ayerst to accelerate the attainment of this goal. Significant progress has been made in the development of a subgroup A RSV vaccine. Essential to the success of this program has been the chimpanzee which responds to RSV infection in a manner most similar to that of susceptible infants. This means that evaluation of live RSV vaccine candidates for attenuation, immunogenicity and protective efficacy in susceptible chimpanzees provides the most relevant assessment of these virus preparations prior to initiation of clinical trials.

A partially attenuated cold-passaged (*cp*) RSV (designated *cp*RSV) was further attenuated by the introduction of additional attenuating mutations, specifically temperature sensitive (*ts*) mutations. Several *cpts* candidate vaccines were found to be satisfactorily attenuated, immunogenic, stable genetically, and protective in chimpanzees. Importantly, these vaccines were shown to be protective in chimpanzees passively infused with RSV immune globulin to achieve a level of RSV neutralizing antibodies equivalent to that present in the target population for RSV vaccine, *i.e.*, very young infants who possess passively-acquired RSV antibodies derived from their mother. Unexpectedly, chimpanzees immunized in the presence of passively transferred RSV antibodies developed unusually high titers of RSV neutralizing antibodies following challenge with wild type virus. These findings are very encouraging and form the basis for ongoing evaluation of several of these vaccine candidates in humans.

Also, wild type and cold-passaged (*cp*) subgroup B viruses have been evaluated for level of replication and immunogenicity in chimpanzees and African green monkeys. Unfortunately, the subgroup B wild type virus does not replicate to high titer in these non-human primates indicating that these animals are not optimal for the evaluation of RSV subgroup B mutants. Nonetheless, the *cp* subgroup B RSV was more restricted in its replication in chimpanzees than its parental wild type virus indicating that the *cp* mutant was attenuated. Evaluation of this candidate mutant in humans will begin shortly.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00326-13 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of Respiratory Viruses in Volunteers

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Brian R. Murphy, M.D.	Head, RV Section	LID, NIAID
Others:	E. Kanta Subbarao, M.D.	Senior Staff Fellow	LID, NIAID
	Susan L. Hall, Ph.D.	Senior Staff Fellow	LID, NIAID
	James E. Crowe, Jr., M.D.	Research Associate	LID, NIAID

COOPERATING UNITS (if any)

PRI DynCorp., Rockville, MD (Potash); Johns Hopkins University, Baltimore, MD (Clements); St. Louis University, St. Louis, MO (Belshe); Vanderbilt University, Nashville, TN (Wright); Wyeth-Ayerst Research, Radnor, PA (Zajac)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Respiratory Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.25

PROFESSIONAL:

0.25

OTHER:

1.00

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A cold adapted influenza A reassortant virus vaccine was shown to be poorly immunogenic at a dose of  $10^6$ TCID<sub>50</sub> in infants less than six months of age, whereas it was highly immunogenic in slightly older children. These results indicate that young age decreases the response to the hemagglutinin of the influenza A virus and suggest that higher doses of vaccine and a multi-dose schedule of immunization will be required for successful immunization in this age group. Such studies are in progress.

The bovine parainfluenza virus type 3 (BPIV3) vaccine was found to be satisfactorily attenuated, stable genetically, poorly transmissible, and immunogenic in seronegative infants and young children. Studies of this promising candidate in the target population of the very young seronegative infant and in older seronegative infants and children are in progress.

The cp45 human PIV3 vaccine also was found to be safe, satisfactorily infectious, genetically stable, and immunogenic in seronegative infants and children. A CRADA is being developed to accelerate evaluation of this promising candidate vaccine strain.

Live attenuated respiratory syncytial virus (RSV) vaccine candidates are currently being evaluated in Phase I trials in infants and young children. One subgroup A virus vaccine that contains both host-range and ts mutations, namely RSV A2 cps-248/955, is infectious in young seropositive vaccinees and, importantly, the ts phenotype of the virus shed is stable. This vaccine is currently being studied in seronegative infants and children. The evaluation of three to four other candidate subgroup A and B vaccine candidates will begin this coming year.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00327-13 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Laboratory Studies of Parainfluenza Type 3 Virus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Susan L. Hall, Ph.D.

Senior Staff Fellow

LID, NIAID

Others: Brian R. Murphy, M.D.

Head, RV Section

LID, NIAID

Peter L. Collins, Ph.D.

Tenured Microbiologist

LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Respiratory Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.25

PROFESSIONAL:

1.25

OTHER:

1.00

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Two live attenuated PIV3 candidate vaccines are in Phase 1-2 trials in human infants and children, namely the cold-passaged 45 mutant of the JS wildtype human PIV3 and the antigenically related bovine PIV3.

A complete cDNA copy of the JS strain of human PIV3 has been constructed. This cDNA has the exact coding sequence of the wild type virus except for engineered mutations that will permit the transfectant virus to escape neutralization by monoclonal antibodies that neutralize the infectivity of the helper virus. With this cDNA, it is now possible in the coming year to attempt to rescue infectious virus from RNA generated from this cDNA.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00345-13 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fill on one line between the borders.)

Laboratory Studies of Respiratory Syncytial Virus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Brian R. Murphy, M.D. Head, RV Section LID, NIAID

Others: James Crowe, M.D. Research Associate (CO) LID, NIAID  
 Robert M. Chanock, M.D. Chief LID, NIAID  
 Peter Collins, Ph.D. Microbiologist LID, NIAID  
 Arun Kulkarni, Ph.D. Visiting Fellow LID, NIAID  
 Jinlin Du, M.D. Visiting Fellow LID, NIAID

COOPERATING UNITS (if any)

LIP/NIAID/NIH (Morse); Wyeth-Ayerst Research, Radnor, PA (Lubeck); The Scripps Research Institute (Burton); PRI, La Jolla, CA (Zebede)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Respiratory Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.25

PROFESSIONAL:

1.25

OTHER:

1.00

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Significant progress has been made in the development of a live attenuated bivalent vaccine against RSV by introducing additional attenuating mutations into mutants that were not completely attenuated for seonegative humans. Several promising candidate RSV subgroup A vaccine candidates have been produced and evaluated. The *cpts* 248/955, 248/404, 530/1009, and 530/1030 mutants were selected from a large panel of mutants for further study because they are attenuated, stable genetically, immunogenic, and efficacious in rodents or chimpanzees. Importantly, these mutants are able to induce a high level of resistance to wild type virus challenge even in chimpanzees passively infused with RSV antibodies at the time of immunization, a situation that simulates that of the human infant with passively acquired RSV antibodies. Progress toward the development of a subgroup B RSV vaccine has also been made this year. We have demonstrated that the *cp*RSV B1/2B5 candidate vaccine virus sustained three independent mutations that contribute to its attenuation phenotype for cotton rats. The attenuation phenotype of this mutant is highly stable even after prolonged replication in immunosuppressed cotton rats. Both components of a bivalent subgroup A and B vaccine were able to replicate *in vivo* without apparent interference. Studies with the *cpts*-248/955 vaccines have been initiated in humans.

The CD8+ T-cell epitope, amino acids 82-90, of the M2 protein of RSV was shown to be the sole mediator of resistance induced by immunization of BALB/c mice with a vaccinia recombinant expressing the M2 protein.

A neutralizing human monoclonal Fab specific for the RSV fusion glycoprotein exhibited significant therapeutic efficacy in RSV-infected mice when introduced directly into the lower respiratory tract at the height of RSV replication in this site. The observed therapeutic efficacy of RSV human monoclonal antibody Fabs generated by antigen selection from a random combinatorial library and produced in *E. coli*, signals a major advance in the immunotherapy of viral respiratory tract disease.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00368-12 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural Analysis of the Genome of Respiratory Syncytial Virus (RSV)

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Peter L. Collins, Ph.D.	Supervisory Microbiologist	LID, NIAID
Others:	Haim Grosfeld, Ph. D.	Visiting Associate	LID, NIAID
	Lili Kuo, Ph.D.	Visiting Fellow	LID, NIAID
	Juan Cristina, Ph.D.	Visiting Fellow	LID, NIAID
	Prabha Atreya, Ph.D.	Visiting Fellow	LID, NIAID
	Siba Samal, Ph.D.	Special Volunteer	LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Respiratory Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.75

PROFESSIONAL:

2.25

OTHER:

0.50

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previously, cDNAs were constructed to encode various truncated versions of the genome RNA of respiratory syncytial virus (RSV) in which the genome termini and putative transcription signals were retained but the viral genes were deleted and replaced with the reporter gene for bacterial chloramphenicol acetyl transferase (CAT). When introduced into RSV-infected cells, the prototype RSV-CAT minigenome appeared to be replicated, transcribed and packaged into virus-like particles. This represents the first type of system in which genome-like RNAs of a nonsegmented negative strand RNA virus were introduced into the viral replication cycle and rendered biologically active. It is an important step in developing the technology for rescuing complete replication-competent virus from cDNA. This capability would allow the engineering of defined viruses as attenuated vaccines and as reagents for molecular biological and viral pathogenesis studies. In its present form, the RSV-CAT system was used to identify and characterize, for the first time, the *cis*-acting replication and transcription signals in the RSV genome. It also should be possible to adapt the RSV-CAT system for the analysis of protein function (accompanying report).





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00372-12 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Roles of RSV Proteins in Host Immunity and Molecular Approaches to Vaccine Design

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Peter L. Collins, Ph.D. Tenured Scientist LID, NIAID

Others: Lili Kuo, Ph.D. Visiting Fellow LID, NIAID  
 Prabha Atreya, Ph.D. Visiting Fellow LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Respiratory Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.25

PROFESSIONAL:

0.75

OTHER:

0.50

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this project is to produce infectious RSV from cDNA. This would make it possible to produce and characterize attenuated strains by direct genetic engineering. To date, we have successfully "rescued" a cDNA-encoded genome analog which is 49.3% of full-length and includes a foreign reporter gene under the control of RSV transcriptive signals. Standard infectious RSV was used as helper to provide proteins to complement the cDNA-encoded genome analog. This analog was packaged into infectious particles and was passaged five times in culture with undiminished efficiency. This provides support for the idea that a complete, nondefective cDNA-encoded genome can be rendered biologically active so as to yield infectious virus. Work towards rescuing a complete infectious virus is described.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00498-08 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Synthesis, Processing and Functions of the Proteins of Human RSV

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Peter L. Collins, Ph. D.	Supervisory Microbiologist	LID, NIAID
Others:	Haim Grosfeld, Ph.D.	Visiting Associate	LID, NIAID
	Lili Kuo, Ph.D.	Visiting Fellow	LID, NIAID
	Juan Cristina, Ph.D.	Visiting Fellow	LID, NIAID
	Prabha Atreya, Ph. D.	Visiting Fellow	LID, NIAID
	Siba Samal, Ph.D.	Special Volunteer	LID, NIAID

COOPERATING UNITS (If any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Respiratory Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.75

PROFESSIONAL:

1.25

OTHER:

0.50

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We previously developed an experimental "rescue" system for respiratory syncytial virus (RSV) based on a cDNA-encoded "minigenome" bearing a foreign marker gene, such as that encoding chloramphenicol acetyl transferase (CAT). These minigenomes can be rendered competent for transcription, replication and incorporation into virions by complementation with proteins supplied by standard RSV helper (accompanying report). We are now attempting to modify this system such that the helper RSV is replaced by RSV proteins synthesized from vectors. This would allow us to supply the proteins in different combinations and amounts. It would then be possible to identify the viral proteins required for the major steps in the viral replicative cycle, in particular transcription, replication and virion formation. This would assign functions to the viral proteins, a number of which are completely uncharacterized. Also, the system could be used to perform detailed structure-function studies of individual proteins involved in all stages of the replicative cycle and would be the method of choice for rescuing complete infectious virus from cDNA-encoded genome RNA.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00308-07 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

*In Vitro* and *In Vivo* Studies of Hepatitis A Virus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Robert H. Purcell, M.D. Head, HV Section LID, NIAID

Others: Stephen M. Feinstone, M.D. Medical Officer LID, NIAID

COOPERATING UNITS (If any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Hepatitis Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

**INACTIVE 1993 - 1994**



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00311-13 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Search For New Hepatitis Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Robert H. Purcell, M.D.	Head, HV Section	LID, NIAID
Others:	Patrizia Farci, M.D.	Visiting Scientist	LID, NIAID
	Norio Ogata, M.D.	Visiting Scientist	LID, NIAID

COOPERATING UNITS (if any)

Hospital for Sick Children, Toronto, Canada (Phillips); Gene Labs, Inc., Redwood City, CA (Kim); Jefferson Medical College, Philadelphia, PA (Muñoz); National Institute of Virology, Pune, India (Arankalle)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Hepatitis Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.83

PROFESSIONAL:

0.83

OTHER:

1.00

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

In 1989 a possible paramyxovirus etiology for giant cell hepatitis was proposed, based on electron micrographic changes seen in the livers of patients with this disease. Collaborative studies, including attempts to transmit the disease to primates, are in progress.

Patients with thalassemia in Sardinia, Italy, receive monthly blood transfusions as therapy. Many of these patients have developed chronic hepatitis. Most cases could be identified as hepatitis B or hepatitis C, but some lacked markers of either of these viruses. These patients are being studied for evidence of previously unrecognized hepatitis viruses. Similarly, clinical samples from patients with acute or chronic non-A,B,C,D,E hepatitis in the United States are to be studied for biological, serological or molecular evidence of transmissible agents. Patients with fulminant non-A, non-B hepatitis generally do not have evidence of infection with any recognized hepatitis virus. Only about 10% can be identified as having fulminant hepatitis C. The remaining 90% remain a diagnostic enigma and may be infected with one or more previously unrecognized viruses. In collaborative studies, we are attempting to transmit the disease to primates.

Evidence for the existence of an additional water-borne hepatitis virus has come from seroepidemiologic studies of enterically transmitted non-A, non-B hepatitis in India. From 50-100% of hepatitis cases in 16 epidemics of water-borne hepatitis were caused by HEV but 1 water-borne epidemic was caused by neither HAV nor HEV. A hepatitis virus was reported some years ago to be transmissible from a non-A, non-B hepatitis patient to marmoset monkeys. The agent, called the GB agent, could be serially transmitted in marmosets and was partially characterized. However, serologic tests could not be developed and the true origin of the virus was never satisfactorily determined. Previous studies in this laboratory with the GB agent are being reactivated and, in collaborative studies, cloning of the viral genome is being attempted.

The objectives of this project are to identify and characterize new etiologic agents of hepatitis and to develop useful assays for diagnosis of infection and seroepidemiologic studies. A longer term objective is the development of passive and active immunoprophylaxis for these important human pathogens.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00314-13 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Woodchuck Virus: Molecular Biological Studies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Roger H. Miller, Ph.D.	Senior Staff Fellow	LID, NIAID
Others:	Minshu Yu, Ph.D.	Senior Staff Fellow	LID, NIAID
	Robert H. Purcell, M.D.	Head, HV Section	LID, NIAID
	Atshushi Shimoda, M.D.,D.M.S.	Visiting Scientist	LID, NIAID
	Janchivin Oyunbileg, B.S.	Special Volunteer	LID, NIAID

COOPERATING UNITS (if any)

Division of Molecular Virology & Immunology, Georgetown University, Washington, DC (Gerin);  
 New York State College of Veterinary Medicine (Tennant)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Hepatitis Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.50

PROFESSIONAL:

3.33

OTHER:

0.17

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The woodchuck hepatitis virus (WHV) is taxonomically and serologically related to the hepatitis B virus (HBV). Infection with each of these viruses is associated with acute and chronic hepatitis and hepatic cell carcinoma in their respective hosts and these associations appear to be etiological in nature. Thus, WHV infection of woodchucks provides a relevant and convenient model for understanding HBV infections of humans. Five major areas of study were pursued this year. First, we continued our investigations of the WHV X gene and found that this gene is very sensitive to alterations and is essential for viral replication in animal transfection experiments. Second, fine mapping of the X transcript promoter was used to identify the essential nucleotides in this cis-acting element. Third, we extended our previous experiments on the characterization of the bi-directional promoter of WHV. Fourth, progress was made in an attempt to identify a Mongolian marmot hepatitis virus. Fifth, two WHV isolates were shown to develop surface antigenemia and liver tumors at significantly different rates. The latter finding has important implications for understanding the molecular mechanisms involved in viral replication and in the oncogenic potential of hepadnaviruses. Thus, it is now possible to design experiments to understand the mechanism of hepadnavirus oncogenesis.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00530-07 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular and Computer Analysis of the Hepatitis B Virus Genome

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Roger H. Miller, Ph.D. Senior Staff Fellow LID, NIAID

Others: Norio Ogata, M.D., D.M.S. Visiting Scientist LID, NIAID  
Robert H. Purcell, M.D. Head, HV Section LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Hepatitis Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.83

PROFESSIONAL:

0.66

OTHER:

0.17

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Hepatitis B virus (HBV) is the prototype of the hepadnavirus family, which includes hepatotropic viruses that infect at least six separate species. Infection with HBV causes polymorphic liver diseases including acute or fulminant hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Over 300 million people throughout the world are chronically infected with HBV and are at high risk of developing hepatocellular carcinoma, one of the world's most prevalent forms of cancer.

Within the last year we pursued two avenues of investigation. First, we continued our characterization of a putative neutralization escape mutant of HBV that emerged in individuals who were vaccinated against hepatitis B. Two licensed vaccines were investigated for their ability to protect chimpanzees against challenge with the S gene mutant. Both recombinant vaccines provided protection against challenge with the mutant suggesting that properly vaccinated individuals are not at risk of infection by the S gene mutant virus. Second, a bi-directional promoter complex was identified within the X gene of HBV further emphasizing the complex genetic organization of this virus.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00569-05 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Determination of Genetic Markers of Virulence and Adaptation to Cell-Culture of Hepatitis A

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Suzanne U. Emerson, Ph.D.	Microbiologist	LID, NIAID
Others:	Ann Funkhouser, M.D.	Research Associate (CO)	LID, NIAID
	Robert H. Purcell, M.D.	Head, HV Section	LID, NIAID
	Gopa Raychaudhuri, Ph.D.	IRTA Fellow	LID, NIAID
	Tina Schultheiss	Special Volunteer	LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Hepatitis Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

4.66

PROFESSIONAL:

2.83

OTHER:

1.83

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Hepatitis A virus (HAV) is a picornavirus with a single-stranded RNA genome of approximately 7500 nucleotides. The wild-type strain of HAV grows poorly in cell-culture, generally is not cytopathic, and virus yields are low. A cell-culture adapted mutant has been selected which grows significantly more efficiently in cell-culture and which is attenuated for marmosets and chimpanzees. The objectives of this project are to determine the genetic basis for virulence and adaptation to cell culture of HAV in order to develop a strain of HAV suitable for use as an attenuated vaccine. The following advances in our understanding of HAV were made.

In an effort to increase replicative capacity, chimeric viruses were constructed from two or more HAV strains including a virulent human strain, an attenuated strain, a vaccine strain, a cytopathic strain, and a simian strain. The P2 region from a cytopathic strain of HAV was shown to confer the large plaque phenotype but not the lytic phenotype of the cytopathic virus. We also constructed viable HAV mutants under the translational control of the encephalomyocarditis internal ribosome site. These chimeric viruses are being analyzed to determine if inclusion of this efficient control element enhances viral protein production or affects host range.

Chimeric viruses were also constructed for the purpose of defining virulence genes. We identified the 2A gene as a second major HAV/7 determinant of attenuation for marmosets and obtained evidence that mutations in the 2A and 2C genes are almost totally responsible for the attenuation of the virus. A full length infectious cDNA of the attenuated HAV MRC-5 cell adapted vaccine strain was constructed to serve as a genetic repository for the vaccine strain and to permit detailed molecular analysis of the virus it encodes. Finally, evidence was obtained that mutations in the 5' non-coding region of the MRC-5 cell adapted virus attenuate the virus for marmosets.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00570-05 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Biology of Hepatitis C Virus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Roger H. Miller, Ph.D.	Senior Staff Fellow	LID, NIAID
Others:	Robert H. Purcell, M.D.	Head, HV Section	LID, NIAID
	Patrizia Farci, M.D.	Visiting Scientist	LID, NIAID
	Jens Bukh, M.D.	Visiting Associate	LID, NIAID
	Raymond Tellier, M.D.	Special Volunteer	LID, NIAID

COOPERATING UNITS (if any)

National Institute of Health, Tokyo, Japan (Shimizu)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Hepatitis Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.5

PROFESSIONAL:

3.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Hepatitis C virus (HCV) is an important human pathogen that can cause acute and chronic hepatitis, liver cirrhosis and, possibly, hepatocellular carcinoma. This virus accounts for up to 25% of community-acquired hepatitis and over 90% of transfusion-associated hepatitis in the United States. The virus particles contain a positive polarity, single-stranded RNA genome with 5' and 3' noncoding (NC) regions. The core (C), envelope 1 (E1) and envelope 2 (E2) proteins are encoded at the 5' terminus and the nonstructural proteins are encoded at the 3' terminus of the single open reading frame of the genome. Furthermore, the finding of genetic heterogeneity of the HCV genome, especially in the genes encoding the envelope proteins, suggests that there may be heterogeneity similar to that seen in the envelope gene of human immunodeficiency viruses. Such a finding would bode ill for attempts at vaccine development. In this project we have extended our previous sequence analysis of the viral genome to include the core gene, which is the most conserved HCV gene. The phylogenetic analysis of this gene was in agreement with that of the envelope 1 gene, which is highly variable. Additional analysis identified conserved features of the core gene that will be useful in understanding the role of the nucleocapsid protein in viral replication. Other major findings were: (i) identification of HCV RNA in dialysis patients; (ii) prevention of HCV infection in chimpanzees following antibody-mediated neutralization in vitro; and (iii) establishment and characterization of antibody-free prototype strains of the different genotypes of HCV.



PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

*In Vivo and In Vitro Studies of Hepatitis E Virus*

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI:	Suzanne U. Emerson, Ph.D.	Microbiologist	LID, NIAID
Others:	Sergei Tsarev, Ph.D., D.Sci.	Visiting Fellow	LID, NIAID
	Shu-Rong Yin, M.D.	Visiting Fellow	LID, NIAID
	Theo Heller, M.D.	Research Associate	LID, NIAID
	Robert H. Purcell, M.D.	Head, HV Section	LID, NIAID

COOPERATING UNITS (if any)

National Institute of Virology, Pune, India (Arankalle); USUHS, Bethesda, MD (Bryan)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Hepatitis Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

4.75

PROFESSIONAL:

3.25

OTHER:

1.50

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Epidemics of enterically transmitted non-A, non-B hepatitis (hepatitis E) have been reported in Asia, Africa, and North America. Similar cases of sporadic hepatitis, presumed to be hepatitis E, account for up to 90% of reported hepatitis in countries where hepatitis E is endemic. Hepatitis E virus (HEV) has been implicated in fulminant hepatitis of pregnancy. This disease has a 20% fatality rate. That a viral agent was responsible for hepatitis E epidemics was first shown in 1983. On the basis of electron microscopy and molecular characterization it was proposed that HEV belongs to the calicivirus family.

The goal of this project is to define the newly identified hepatitis E virus (HEV), determine the extent and pattern of its involvement in enterically transmitted hepatitis, and to develop a vaccine which prevents hepatitis E. We performed a retrospective seroepidemiologic study which showed that 16 of 17 epidemics of waterborne hepatitis in India were caused by HEV and that one was caused by a previously unrecognized agent. In addition, we showed that the age-specific antibody profile for HEV in Pune, India has not changed over a decade. We also analyzed sera from an epidemic of hepatitis E in Pakistan and obtained evidence that antibody to HEV protects humans against disease. Studies carried out in cynomolgus monkeys demonstrated that passively acquired antibody to HEV protected the animals from disease even when infection was not prevented. Most importantly, we demonstrated that active immunization with a recombinant HEV capsid protein protected cynomolgus monkeys completely against hepatitis E disease and partially against infection.

We constructed a full-length cDNA clone of a Chinese strain of HEV for use in molecular studies. The recombinant ORF-3 protein of HEV was efficiently expressed in insect cells and a recombinant ORF-1 protein was efficiently expressed and secreted from yeast cells. These recombinant proteins are being studied to define the functions of the proteins. In addition they will serve as substrates in ELISA assays which are being developed to increase the sensitivity and reliability of serological assays.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00500-06 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Processing and Immunogenicity of Dengue Type 4 Virus Nonstructural Protein NS1

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Ching-Juh Lai, Ph.D.	Head, MVB Section	LID, NIAID
Others:	Barry Falgout, Ph.D.	Senior Staff Fellow	LID, NIAID
	Lewis Markoff, M.D.	Medical Officer	LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Molecular Viral Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**INACTIVE 1993 - 1994**



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00531-05 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Functional Analysis of Dengue Nonstructural Proteins NS2B and NS3

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI: Ching-Juh Lai, Ph.D. Head, MVB Section LID, NIAID

Others: Barry Falgout, Ph.D. Senior Staff Fellow LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Molecular Viral Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**INACTIVE 1993 - 1994**





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00571-03 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Amino Acid Substitution at the NS1-NS2A Cleavage Junction of Dengue Virus Polypeptide

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Michèle Pethel Microbiologist LID, NIAID

Others: Ching-Juh Lai, Ph.D. Head, MVB Section LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Molecular Viral Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**INACTIVE 1993 - 1994**



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00572-03 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Processing of Dengue Virus Polyprotein NS3-NS4A-NS4B-NS5

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Ching-Juh Lai, Ph.D. Head, MVB Section LID, NIAID

Others: Barry Falgout, Ph.D. Senior Staff Fellow LID, NIAID  
Annie Cahour, Ph.D. Visiting Associate LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Molecular Viral Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**INACTIVE 1993 - 1994**



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00598-02 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Dengue Type 4 Virus Mutants Restricted in Polypeptide Processing

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI: Hiroshi Kawano, M.D.

Visiting Associate

LID, NIAID

Others: Ching-Juh Lai, Ph.D.

Head, MVB Section

LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Molecular Viral Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

**INACTIVE 1993 - 1994**





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AI 00600-04 LID						
PERIOD COVERED October 1, 1993 to September 30, 1994								
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Engineering Viable Dengue Virus 3' Noncoding Region Deletion Mutants for Use in Vaccines</b>								
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%; padding: 5px;">PI: Ruhe Men, M.D.</td> <td style="width: 33%; padding: 5px;">Visiting Associate</td> <td style="width: 33%; padding: 5px;">LID, NIAID</td> </tr> <tr> <td style="padding: 5px;">Others: Ching-Juh Lai, Ph.D.</td> <td style="padding: 5px;">Head, MVB Section</td> <td style="padding: 5px;">LID, NIAID</td> </tr> </table>			PI: Ruhe Men, M.D.	Visiting Associate	LID, NIAID	Others: Ching-Juh Lai, Ph.D.	Head, MVB Section	LID, NIAID
PI: Ruhe Men, M.D.	Visiting Associate	LID, NIAID						
Others: Ching-Juh Lai, Ph.D.	Head, MVB Section	LID, NIAID						
COOPERATING UNITS (if any) Walter Reed Army Institute of Research, Washington, DC (Hoke and Eckels)								
LAB/BRANCH Laboratory of Infectious Diseases								
SECTION Molecular Viral Biology Section								
INSTITUTE AND LOCATION NIAID, NIH, Bethesda, MD 20892								
TOTAL STAFF YEARS:	1.0	PROFESSIONAL:						
	1.0	OTHER:						
		0.0						
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews								
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>           Success in developing methods for introducing site-specific mutations into the dengue type 4 virus (DEN4) genome has provided a new strategy for the construction of safe and effective vaccines against dengue virus infection. A series of cDNA constructs was engineered to contain deletions ranging from 30 to 202 nucleotides in length in the 3' non-coding region of the genome. Full length RNA transcripts of these DNA constructs were tested for infectivity by transfecting permissive tissue culture cells. A panel of viable DEN4 mutants was recovered from mutant RNA transfected cells. Many deletion mutants were stable and produced plaques of reduced size on mosquito C6/36 cells compared to wild type virus. Analysis of the mutants in simian LLC-MK<sub>2</sub> cells revealed that most deletion mutants produced plaques that were slow to develop. Furthermore, most mutants that grew slowly attained a lower titer than parental wild type virus. These observations indicate that these DEN4 deletion mutants were growth-restricted in cell culture. Interestingly, deletion mutant 3'd 303-183 produced small plaques on C6/36 cells but grew to a high titer similar to that of parental wild type virus. This deletion mutant plus four other growth-restricted deletion mutants were selected for evaluation of their infectivity and immunogenicity in rhesus monkeys compared to that of the wild type virus. Monkeys infected with those deletion mutants that were restricted in their growth in cultured cells developed fewer days of viremia compared to monkeys infected with the wild type virus. Mutant 3'd 303-183 induced an antibody response equivalent to that of wild type virus as measured by radioimmunoprecipitation and virus neutralization. In contrast, the other deletion mutants induced a low to moderate level of antibodies compared to parental virus. The immunogenicity of these 3' deletion mutants in monkeys appeared to correlate directly with their growth in LLC-MK<sub>2</sub> cells. Three deletion mutants of DEN4 which induced a moderate to high level antibody response in monkeys were selected for production of candidate vaccine seed lots that will be evaluated in primates. The results of these studies in primates will determine whether clinical trials will be initiated and which mutants will be studied.         </p>								



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00637-03 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of New Approaches to Vaccines Against the Tick-Borne Encephalitis Virus Complex

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Alexander Pletnev, Ph.D., D. Sci. Visiting Scientist LID, NIAID

Others: Ching-Juh Lai, Ph.D. Head, MVB Section LID, NIAID  
Michael Bray, M.D. Senior Staff Fellow LID, NIAID  
Robert M. Chanock, M.D. Chief LID, NIAID

COOPERATING UNITS (If any)

USAMRIID (Huggins)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Molecular Viral Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previously, viable chimeric flaviviruses were constructed that contained tick-borne encephalitis virus (TBEV) structural protein CME or ME genes with the remaining genes derived from dengue type 4 virus (DEN4). The ME chimera retained the neurovirulence for mice of its TBEV parent from which its M and E genes were derived, but it lacked the peripheral invasiveness of TBEV. The ME chimera was subjected to mutational analysis in an attempt to reduce or ablate neurovirulence manifest when virus is inoculated directly into the brain. Three distinct mutations were independently associated with marked reduction of mouse neurovirulence. These mutations ablated: (i) the TBEV PreM cleavage site which is required for proper processing of M protein; (ii) the TBEV E (envelope glycoprotein) glycosylation site; or (iii) the first DEN4 NS1 (non-structural protein one) glycosylation site. Each of the 3 attenuated mutants was restricted in growth in both simian and mosquito cells. Significantly, parenteral inoculation with these attenuated mutants induced complete resistance in mice to fatal encephalitis caused by subsequent challenge with the highly neurovirulent ME chimera. These observations suggest a new strategy for developing a live attenuated TBEV vaccine.

Unlike the highly virulent TBEV, the naturally occurring related Langat virus (LGT) is markedly less pathogenic for mice. Genetic analysis of the LGT may allow us to identify the molecular basis for neuroinvasiveness and neurovirulence of TBE viruses. The RNA genome of LGT (strain TP21) is 10940 nt in length and contains an open reading frame for a polyprotein of 3,414 amino acids. The 5' noncoding region is 129 nt in length of which nts. 1-25 and nts. 80-128 are conserved in the corresponding regions of the related TBEV or Powassan virus (a TBE virus of North America). LGT contains 583 nt in the 3' noncoding region of which the last 90 nt are conserved among viruses of the TBE complex. It may be possible to develop a safe and effective live attenuated TBEV vaccine by constructing DEN4-LGT chimeric viruses that express LGT antigenicity. Attenuating mutations similar to those identified earlier will be introduced in the chimeric virus genome and progeny virus analyzed for immunogenicity and loss of virulence.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00638-03 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Expression of Dengue and Other Flavivirus Proteins Using Baculovirus as a Vector

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Ching-Juh Lai, Ph.D. Head, MVB Section LID, NIAID

Others: Ruhe Men, M.D. Visiting Associate LID, NIAID  
Chun-Fa Zhang, Ph.D. Visiting Associate LID, NIAID

COOPERATING UNITS (if any)

WRAIR, Washington, DC (Summers, Eckels, Dubois, Sumiyoshi)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Molecular Viral Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**TERMINATED 1993 - 1994**





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AI 00681-02 LID
PERIOD COVERED October 1, 1993 to September 30, 1994		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Determination of Genetic Loci of Dengue Type 2 Virus Neurovirulence in Mice		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Michael Bray, M.D.	Senior Staff Fellow LID, NIAID
Others:	Ching-Juh Lai, Ph.D.	Head, MVB Section LID, NIAID
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Infectious Diseases		
SECTION Molecular Viral Biology Section		
INSTITUTE AND LOCATION NIAID, NIH, Bethesda, MD 20892		
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>           The four serotypes of dengue virus cause an estimated 100-200 million cases human disease in tropical regions each year. Infection with one serotype produces durable immunity only to that type. Serotype is conferred by the viral structural proteins, capsid (C), pre-membrane (pre-M) and envelope (E). The dengue viral genome, an 11 kilobase strand of positive-sense RNA, contains the three structural protein genes followed by a series of 7 non-structural protein genes. Earlier, a full-length cDNA copy of the entire dengue type 4 virus (D4) genome was constructed. Transfection of mammalian cells with RNA transcripts from this template yielded D4 virus, designated D4 2A. We subsequently replaced the C-preM-E or preM-E genes of the full-length clone with the structural protein genes of two different strains of dengue type 2 virus (D2). In one construct the D4 preM-E genes were replaced with the preM-E genes of a D2 New Guinea C strain virus isolated from a human patient (D2 NGC parental) and in the other the C-preM-E or preM-E genes of the mouse-neurovirulent mutant selected from the parental virus during serial passage in mouse brain, i.e., D2 NGC neurovirulent. Neither D4 2A nor the chimeric virus (D2 ME parental)/D4 was neurovirulent in suckling mice, whereas chimeric viruses containing the C-preM-E or preM-E genes of D2 NGC neurovirulent produced fatal encephalitis. It appears that some or all of the genetic loci responsible for neurovirulence are located in the structural protein genes. Sequence analysis of the cloned C-preM-E genes of parental and neurovirulent D2 NGC identified 7 mutations in the neurovirulent mutant which result in an amino acid change. Based on this information a series of chimeric viruses were constructed in which single or multiple mutations in pre-M and/or E were substituted into the parental sequence, and the resulting mutants were tested for neurovirulence in mice. A mutation in the N-terminal one-third of E protein which changed the negatively charged glutamic acid in the parental E protein to a positively charged lysine appeared to be sufficient to produce the neurovirulence phenotype.         </p>		





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00682-02 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fill on one line between the borders.)

Evaluation of Intertypic Chimeric Dengue Viruses as Candidate Vaccines

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Michael Bray, M.D. Senior Staff Fellow LID, NIAID

Others: Ching-Juh Lai, Ph.D. Head, MVB Section LID, NIAID

COOPERATING UNITS (If any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Molecular Viral Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The dengue viral genome, an 11 kb strand of positive-sense RNA, contains genes for the three structural proteins, capsid (C), pre-membrane(pre-M) and envelope (E), followed by a series of 7 non-structural protein genes. Earlier, we constructed a full-length cDNA copy of the entire dengue 4 (D4) genome, which yields infectious full-length RNA transcripts. Subsequently the C-preM-E genes of the full-length clone were replaced with the C-preM-E genes of the Western Pacific strain of D1 virus or the C-preM-E or preM-E genes of the parental New Guinea C strain of D2 virus, and used as templates to create chimeric D1/4 and D2/4 viruses. Cells infected with these chimeras produced the appropriate authentic D1 or D2 structural proteins. Two chimeric viruses were tested in rhesus monkeys to determine their potential usefulness as vaccines. Initially, monkeys were immunized with either D4 virus, wild-type D1 or D2, or chimeric D1/4 or D2/4 virus. Monkeys inoculated with D4 developed minimal viremia and were not protected against D1 or D2 challenge. Those given D1 developed 2-3 days of viremia, had a high-titer neutralizing antibody response, and were protected against subsequent D1 challenge. D1/4 recipients had little (1 day) or no viremia; three had a strong D1 neutralizing antibody response and developed little (1 day) or no viremia following subsequent D1 challenge, while the fourth monkey had a weak antibody response to immunization and had 4 days of viremia post-challenge. Each of the monkeys infected with D2 or D2/D4 developed viremia (4-6 days vs. 1-5 days, respectively). Each monkey developed a high-titer D2 antibody response and were completely protected against D2 challenge. In a second experiment, monkeys were immunized with D4 or with a mixture of the chimeric D1/D4 and D2/D4 viruses, as a prototype bivalent vaccine. The D4 recipients became viremic when challenged subsequently with D1 or D2, but each of the monkeys immunized with D1/4 plus D2/4 developed high-titer neutralizing antibodies against both D1 and D2 and were protected against challenge with D1 or D2. Our results suggest that a mixture of suitably engineered chimeric viruses might serve together as a safe and effective live dengue vaccine.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00683-02 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic Determinants of Dengue Virus Mouse Neurovirulence

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Ching-Juh Lai, Ph.D. Head, MVB Section LID, NIAID

Others: Kazufumi Hiramatsu, M.D. Visiting Associate LID, NIAID  
 Weiran Chen, Ph.D. Visiting Fellow LID, NIAID

COOPERATING UNITS (if any)

University of Hawaii, Honolulu, HA (Rosen)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Molecular Viral Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Shortly after the first strains of dengue virus were isolated in the laboratory, serial intracerebral passage of dengue type 1 or type 2 virus (DEN1 or DEN2) in mice was employed to attenuate these viruses for use as live attenuated vaccines in humans. Studies by Sabin revealed that at a low passage level these viruses had lost most of their virulence. In addition, a highly neurovirulent mutant of the non-pathogenic parental DEN4 strain H241 was selected by serial intracerebral passage in mice. The genetic basis for neurovirulence of the DEN4 mouse-adapted mutant was studied by comparing intratypic chimeric viruses that contained the three structural protein genes of the parental virus or its neurovirulent mutant on the background sequence of non-neurovirulent DEN4 strain 814669. The chimera that contained the three structural protein genes of mouse neurovirulent DEN4 H241 was highly neurovirulent in mice, whereas the chimera that contained the corresponding genes of its non-mouse adapted parent was not neurovirulent. Thus, some or all of the genetic loci for neurovirulence of the DEN4 mutant map within the structural protein genes. The parent and its mouse neurovirulent mutant were found to differ by only five amino acid differences in their structural proteins. Three of the mutations were located in the envelope (E) glycoprotein. Two of these amino acid changes were identified as important determinants of DEN4 mouse neurovirulence: (i) the single substitution of Ile for Thr<sub>434</sub> which ablated one of the two conserved glycosylation sites in DEN4 E yielded a virus that was almost as neurovirulent as the mouse-adapted mutant; and (ii) the Leu for Phe<sub>680</sub> substitution also yielded a neurovirulent virus but it was less neurovirulent than the glycosylation mutant. The parental DEN1 Hawaii strain and its mouse neurovirulent mutant were also studied in an attempt to map the DEN1 genetic loci responsible for mouse neurovirulence. Thirteen amino acid differences in the C-PreM-E structural protein region were identified. Among the 13 changes 7 are located in E, 3 in PreM and 3 in C.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00706-01 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic Loci Responsible for Growth Restriction of Mouse-Adapted Dengue Viruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Ching-Juh Lai, Ph.D. Head, MVB Section LID, NIAID

Others: Ruhe Men, Ph.D. Visiting Scientist LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Molecular Viral Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Serial intracerebral passage of dengue type 1 and type 2 viruses in mice was shown previously to attenuate these viruses for humans. Dengue type 4 virus strain H241 was also successfully adapted to replicate in mouse brain. The mouse-adapted DEN4 H241N was highly neurovirulent, whereas its parent DEN4 H241P was not. In addition, DEN4 H241N replicated less efficiently than DEN4 H241P in simian LLC-MK<sub>2</sub> cells. An intratypic DEN4 chimera containing the C-PreM-E structural protein genes from DEN4 H241N also exhibited marked restriction of growth in LLC-MK<sub>2</sub> cells. Analysis of viral proteins produced in LLC-MK<sub>2</sub> cells by DEN4 H241N or its derived C-PreM-E chimera indicated that very little PreM was produced and that which was detected migrated slightly slower than the PreM of DEN4 H241P or its chimeric derivative. Recent evidence indicates that immature PreM-containing flaviviruses replicate less efficiently than the mature M-containing virus. Studies were performed to determine whether the altered PreM or mutations in C or E might affect the normal processing of PreM to produce M normally present in the mature virion. Protein analysis indicated that DEN4 E, PreM, M and C were detected in the virion preparation of DEN4 H241P or its derived chimera. On the other hand, the virion preparation of DEN4 H241N or its derived chimera contained E, PreM and C, but M was not detected. This suggested that cleavage of PreM to M was defective for DEN4 H241N and the genetic loci for the defect mapped within the C-PreM-E genes. There were six amino acid differences in the structural protein gene region between DEN4 H241P and DEN4 H241N: 1 in C, 2 in PreM and 3 in E. To identify mutations responsible for the defective PreM cleavage, 8 chimeric mutants were constructed that contained one or more amino acid substitutions that are present in the mutant C, PreM or E. Only mutant DEN4(H241P, S456) which contained all three amino acid substitutions in E exhibited the PreM cleavage defect. Interestingly, chimeric mutants which contained both mutations in PreM processed PreM normally. This suggests that PreM interacts with E during virus maturation.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00333-13 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Longitudinal and Cross-Sectional Studies of Viral Gastroenteritis in Infants and Young Children

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Albert Z. Kapikian, M.D. Head, Epid. Section LID, NIAID

Others: Kim Y. Green, Ph.D. Senior Staff Fellow LID, NIAID  
 Judy F. Lew, M.D. Senior Research Invest. LID, NIAID  
 Mariam W. Watson, B.S. Biologist LID, NIAID

COOPERATING UNITS (if any)

Children's Hospital National Medical Center, Washington, DC (Kim); University of Massachusetts Medical School, Worcester, MA (Herrman and Blacklow); John Radcliffe Hospital, Oxford, United Kingdom (Kurtz and Lee)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

<0.1

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

An important unresolved area of pediatric research involves the role of various viral agents in the etiology of acute gastroenteritis. Although the importance of rotaviruses is firmly established, the role of other viruses such as the astroviruses and caliciviruses has not been resolved. The goal of this project is to place these viruses in perspective with regard to their relative contribution to various forms of acute gastroenteritis in infants and young children. The availability of stool and serum specimens from several large-scale pediatric studies places us in the enviable position of being able to address these issues. Two major studies provide the focus of this project. One is a longitudinal study (1955-1969) at Junior Village, a welfare institution for homeless but otherwise normal children, and the other a cross-sectional study (1974-1991) of children hospitalized with gastroenteritis at Children's Hospital National Medical Center, Washington, DC. Our goal in the Junior Village studies has been to investigate the natural history of calicivirus and astrovirus infections in a longitudinal setting, whereas the Children's Hospital study provides materials that should allow us to determine the importance of calicivirus and astrovirus as agents of severe gastroenteritis requiring admission to the hospital. There is evidence from several studies including this one that the Norwalk viruses (now classified as caliciviruses) and astroviruses both cause infection in infants but, as yet, their importance as etiologic agents of severe gastroenteritis is not certain. This type of information must be obtained before priorities for vaccine development can be set.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00339-13 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Isolation and Serotypic Characterization of Human and Animal Rotaviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Yasutaka Hoshino, D.V.M. Visiting Scientist LID, NIAID

Others: Albert Z. Kapikian, M.D. Head, Epid. Section LID, NIAID

COOPERATING UNITS (if any)

University of Pavia, Italy (Gerna); University of Nebraska (Duhamel)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.50

PROFESSIONAL:

0.25

OTHER:

1.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The human rotavirus strain PA169 (VP7 serotype 6) which was originally isolated from a child with diarrhea was found to possess a new VP4 neutralization specificity distinct from ten known VP4 serotypes. This is the sixth human rotavirus VP4 serotype. The bovine rotavirus strain NCDV-Cody which was originally isolated from a diarrheic calf and traditionally considered as the virulent counterpart for the attenuated NCDV-Lincoln (VP7 serotype 6) was shown to belong to VP7 serotype 8.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00340-13 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic Studies of Rotavirus Pathogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Yasutaka Hoshino, D.V.M.	Visiting Scientist	LID, NIAID
Others:	Ronald Jones	Biologist	LID, NIAID
	Albert Z. Kapikian, M.D.	Head, Epid. Section	LID, NIAID

COOPERATING UNITS (if any)

Ohio State University (OARDC), Wooster, Ohio (Saif)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

<0.1

PROFESSIONAL:

<0.1

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previously, by using a semi-homologous system of gnotobiotic newborn pigs and virulent porcine rotavirus (strain SB-1A) and avirulent human rotavirus (strain DS-1) and their reassortants, we demonstrated that: (i) the 3rd (VP3), 4th (VP4), 9th (VP7), or 10th (NS28) porcine rotavirus gene each play an important independent role in virulence of rotavirus infection in piglets, and (ii) all four of the porcine rotavirus virulence-associated genes are required for the induction of diarrhea and the shedding of virus by piglets. These observations suggested a new potential strategy for attenuation of wild-type human rotaviruses of major epidemiologic importance and the subsequent development of a safe and effective vaccine.

Based on this strategy efforts were made to generate four human x bovine rotavirus reassortants, each of which has: (i) the VP4-encoding gene from human rotavirus Wa (VP4:1A); (ii) the VP7-encoding gene from human rotavirus D (VP7:1), DS-1 (VP7:2), P (VP7:3), or ST3 (VP7:4); and (iii) the remaining 9 genes including the VP3-encoding gene and NS28-encoding gene from bovine rotavirus UK. In addition, we successfully generated two human x bovine rotavirus reassortants, each of which had the VP4-encoding gene from human rotavirus Wa (VP4:1A) or DS-1 (VP4:1B) and the remaining 10 genes from bovine rotavirus UK.



PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Evaluation of Live Attenuated Rotavirus Vaccines

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Albert Z. Kapikian, M.D.	Head, Epid. Section	LID, NIAID
Others:	Yasutaka Hoshino, D.V.M.	Visiting Scientist	LID, NIAID
	Kim Y. Green, Ph.D.	Senior Staff Fellow	LID, NIAID
	Robert M. Chanock, M.D.	Chief	LID, NIAID

COOPERATING UNITS (if any)

Institute of Biomedicine, Caracas, Venezuela (Pérez-Schael; Rojas); Univ. of Rochester (Treanor); Johns Hopkins University (Midthun; Clements); Univ. of Tampere (Vesikari, Ruuska); Institute of Nutrition, Peru (Lanata); Wycht-Ayerst Research; Secretech, Inc. (Schafer); Dyn-Corp-PRI (Potash)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.00

PROFESSIONAL:

0.75

OTHER:

1.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Rotaviruses are the single most important cause of severe diarrhea in infants and young children in both developed and developing countries, accounting for 30-50% of such illnesses. The "Jennerian" approach to vaccination, which involves the use of an attenuated, antigenically-related live virus strain derived from a non-human host, has been evaluated in clinical trials using surrogate rotavirus strains of bovine origin by others or of rhesus monkey origin by us. This approach has had limited success because serotype-specific immunity against each of the four clinically important human rotavirus VP7 serotypes could not be achieved consistently in infants less than six months of age with such an orally administered monovalent vaccine. We, therefore, developed a modified "Jennerian" approach that involved formulation of a quadrivalent rotavirus vaccine containing: (i) rhesus rotavirus (RRV) (VP7 serotype 3), and (ii) three human rotavirus-RRV reassortants, each possessing ten RRV genes and a single human rotavirus gene that encodes VP7 serotype 1, 2, or 4 specificity. Placebo-controlled field trials of individual reassortant vaccines or the quadrivalent vaccine involving 8600 infants and young children in the United States or overseas have been completed, are in progress or are under analysis. The modified strategy developed in LID appears to be quite promising, especially with regard to preventing the the most serious consequences of rotavirus infection, namely severe diarrhea. A significant reduction in the incidence of rotavirus diarrhea of any severity was observed in the two most, recent large clinical trials in the US. Protective efficacy was 63% and 49%, respectively (Sack *et al.* and Dennehy *et al.*, Abstracts). Of particular significance was the observation that the quadrivalent LID vaccine was even more effective against severe rotavirus diarrhea, as 80% protective efficacy was achieved in the second study.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00342-13 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Gastroenteritis Viruses by Electron Microscopy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Albert Z. Kapikian, M.D. Head, Epid. Section LID, NIAID

COOPERATING UNITS (if any)

U.S. Naval Medical Research Institute, Bethesda, MD (Savarino, Sharp); Vanderbilt University (Wright)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.55

PROFESSIONAL:

0.30

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Although the electron microscope has been used steadily by our laboratory, its peak activity occurred about two decades ago when it was the vehicle for: (i) the discovery of the first important gastroenteritis virus, the Norwalk virus, followed in succession by the discovery of the Hawaii virus and Montgomery County virus, other agents of viral gastroenteritis; (ii) the discovery of the hepatitis A virus; (iii) the initial detection in the United States of human rotavirus (which was discovered in Australia), the most important cause of severe diarrhea of infants and young children; and (iv) understanding the natural history and importance of rotaviruses in infants and young children. In the past decade it was also instrumental in studies of hepatitis E virus. This past year marked a striking resurgence of activity involving the electron microscope because it proved to be: (i) the only method for detecting recombinant Norwalk, Desert Shield, Hawaii and Toronto virus-like particles that were expressed in insect cells; and (ii) the only method to demonstrate specific antigenic relationships among these recombinants (by immune electron microscopy). As a result, the electron microscope has been an important adjunct to many of the projects of the section providing seminal information in the progress of molecular biologic (as well as other) studies. The scope of the use of the electron microscope is evidenced by the observation that over 100 individual experiments were conducted by electron microscopy since the previous annual report.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00343-13 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of Norwalk Virus and Related Norwalk-Like Viruses in Viral Gastroenteritis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Albert Z. Kapikian, M.D. Head, Epid. Section LID, NIAID

Others: Kim Y. Green, Ph.D. Senior Staff Fellow LID, NIAID  
Judy Lew, M.D. Senior Research Invest. LID, NIAID

COOPERATING UNITS (if any)

Baylor College of Medicine (Estes); U.S. Naval Medical Research Institute (Hyams, Sharp, Savarino); University of Tampere, Finland (Vesikari)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.45

PROFESSIONAL:

0.20

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Norwalk virus and related 27nm viruses are an important cause of epidemic viral gastroenteritis that occurs in family, school, group, institutional or community-wide outbreaks affecting adults, school-aged children, family contacts and some young children as well. However, large scale epidemiologic studies as well as the development of routine laboratory diagnostic assays for Norwalk virus have been hindered by the absence of a readily accessible source of antigen because this virus has not yet been grown in any tissue culture system. Therefore, serologic assays have relied on antigen positive stool material. Recently, this obstacle was overcome by the production of recombinant Norwalk virus capsid protein in a baculovirus expression system (Estes), and this self-assembled virus-like particle was found to be comparable to the native virus in serologic assay. We have used the recombinant Norwalk virus antigen in an ELISA to examine the serologic responses to Norwalk virus in various populations. We have previously shown that: (i) Norwalk virus infection occurred in 6.2% of a subset of troops deployed to Saudi Arabia and Kuwait during a five-month interval; and (ii) over a period of almost 2 years, 49% of 154 infants and young children in Finland exhibited serologic evidence of Norwalk virus infection. During this reporting period, we describe the use of the recombinant Norwalk virus antigen in: (i) elucidating the role of Norwalk virus infection in an outbreak of gastroenteritis aboard a U.S. aircraft carrier, and (ii) among troops who developed gastroenteritis while deployed to Somalia.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00446-09 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Testing of Rotavirus Vaccine Candidates in Venezuela and Peru

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI: Jorge Flores, M.D.

Visiting Scientist

LID, NIAID

Others: Albert Z. Kapikian, M.D.

Head, Epid. Section

LID, NIAID

COOPERATING UNITS (if any)

Instituto de Biomedicina, Caracas, Venezuela (Pérez-Schael); Instituto de Investigacion Nutricional, Lima, Peru (Lanata)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

**TERMINATED 1993 - 1994**





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00451-08 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunological and Molecular Characterization of Rotavirus Antigens

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Albert Z. Kapikian, M.D. Head, Epid. Section LID, NIAID

Others: Mario Gorziglia, Ph.D. Visiting Associate LID, NIAID  
Koki Taniguchi, Ph.D. Visiting Associate LID, NIAID  
Kazuo Nishikawa, M.D. Visiting Associate LID, NIAID  
Kim Green, Ph.D. Staff Fellow LID, NIAID  
Yasutaka Hoshino, D.V.M. Visiting Scientist LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

**TERMINATED 1993 - 1994**



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00507-07 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Characterization of Rotavirus Serotypes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kim Y. Green, Ph.D.

Senior Staff Fellow

LID, NIAID

COOPERATING UNITS (If any)

University of Rochester, Rochester, NY (Madore & Dolin); University of Tampere, Tampere, Finland (Vesikari)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**INACTIVE 1993 - 1994**



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00533-06 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analysis of Rotavirus Proteins with Monoclonal Antibodies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kim Y. Green, Ph.D. Senior Staff Fellow LID, NIAID

Others: Albert Z. Kapikian, M.D. Head, Epid. Section LID, NIAID

COOPERATING UNITS (if any)

University of Tampere, Tampere, Finland (Vesikari)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

**INACTIVE 1993 - 1994**



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00534-06 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Analysis of Non-Group A Rotaviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kim Y. Green, Ph.D.

Senior Staff Fellow

LID, NIAID

COOPERATING UNITS (if any)

Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio (Saif); Capital Institute of Pediatrics, Beijing, China (Qian)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**INACTIVE 1993 - 1994**





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00573-04 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Expression of Rotavirus Proteins in *Salmonella* Bacteria

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kim Y. Green, Ph.D. Senior Staff Fellow LID, NIAID

COOPERATING UNITS (if any)

University of Maryland, Baltimore, Maryland (Levine & Hone); Washington University (Curtiss);  
University of Missouri (Parker)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects   ☐ (b) Human tissues   ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

INACTIVE 1993 - 1994



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00574-04 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Expression and Distribution of Conserved and Serotype-Specific Epitopes on Rotavirus VP8

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Mario Gorziglia, Ph.D.

Visiting Associate

LID, NIAID

Others: Mitzi Sereno  
Gisela Larralde

Biologist  
Guest Researcher

LID, NIAID  
UCV, Venezuela

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

TERMINATED 1993 - 1994



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00576-04 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Serotype Analysis and Characterization of Rotaviruses from Malaysia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Nassar Rasool, Ph.D.

Guest Researcher

LID, NIAID

Others: Mario Gorziglia, Ph.D.  
Albert Z. Kapikian, M.D.

Visiting Associate  
Head, Epid. Section

LID, NIAID  
LID, NIAID

COOPERATING UNITS (If any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

**TERMINATED 1993 - 1994**





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00601-02 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of Recombinant BCG-VP8 Vaccines

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Mario Gorziglia, Ph.D. Visiting Associate LID, NIAID

Others: Mitzi Sereno Biologist LID, NIAID  
Yasutaka Hoshino, D.V.M. Visiting Scientist LID, NIAID

COOPERATING UNITS (If any)

MedImmune Laboratory (Stover)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**TERMINATED 1993 - 1994**



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00602-02 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Expression of Rotavirus Outer Capsid Protein VP4 by Recombinant-Adenovirus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Mario Gorziglia, Ph.D. Visiting Associate LID, NIAID

Others: Albert Z. Kapikian, M.D. Head, Epid. Section LID, NIAID

COOPERATING UNITS (If any)

Wyeth-Ayerst Research Laboratories (Davis & Selling)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

**TERMINATED 1993 - 1994**



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00603-03 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Monoclonal Antibodies Directed to VP8 Subunit of VP4

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Mario Gorziglia, Ph.D.

Visiting Associate

LID, NIAID

Others: Ferdinando Liprandi

Guest Researcher

IVIC, Venezuela

COOPERATING UNITS (if any)

UCV, Venezuela (Larralde)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**TERMINATED 1993 - 1994**



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00604-04 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cold-Adaptation of Human Rotaviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Yasutaka Hoshino, D.V.M. Visiting Scientist LID, NIAID

Others: Albert Z. Kapikian, M.D. Head, Epid. Section LID, NIAID  
Robert M. Chanock, M.D. Chief LID, NIAID

COOPERATING UNITS (if any)

DynCorp (Potash)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.10

PROFESSIONAL:

0.75

OTHER:

0.35

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Virulent, wild-type human rotaviruses of major epidemiological importance were subjected to passage in cell culture at suboptimal temperature in an attempt to isolate cold-adapted mutants that were attenuated for humans and suitable for use in a live viral vaccine. Mutants of each of these rotaviruses were selected during successive serial passage in primary African green monkey kidney cells at progressively lower suboptimal temperature (30°C, 28°C, and 26°C). The mutants exhibited both temperature sensitivity of plaque formation (*i.e.*, a *ts* phenotype) and ability to form plaques efficiently at suboptimal temperature as compared to parental wild-type rotavirus (*i.e.*, a cold-adaptation [*ca*] phenotype). A detailed analysis of viruses recovered after the individual passages in the 30°C series indicated that the level of cold-adaptation observed at the end of the series was the result of two to three discrete incremental increases in ability to replicate and form plaques at 30°C. The succeeding set of ten serial passages at 28°C selected mutants that exhibited a greater degree of cold-adaptation and three of the mutants exhibited an associated increase in temperature sensitivity. Finally, in the case of three of the strains, the third successive serial passage series which was performed at 26°C selected for mutants with an even greater degree of cold-adaptation that was associated with greater temperature sensitivity in one instance. This suggested that each of these viruses sustained a minimum of four to five mutations during the total selection procedure. These mutations imposed a varying degree of growth restriction at physiologic temperatures (*i.e.*, 36°-39°C) *in vitro*. Recovery of mutants exhibiting a relatively wide range of growth restriction *in vitro* provides a collection of viruses that can be searched for promising live vaccine candidate strains. The availability of such a disparate set of mutants should increase the likelihood of identifying promising candidate vaccine mutants.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00605-04 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Experimental Infection of Chimpanzees by Human Rotavirus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Albert Z. Kapikian, M.D. Head, Epid. Section LID, NIAID

Others: Yasutaka Hoshino, D.V.M. Visiting Scientist LID, NIAID  
 Eileen N. Ostlund, D.V.M. IRTA Fellow LID, NIAID  
 Robert M. Chanock, M.D. Chief LID, NIAID

COOPERATING UNITS (if any)

Georgetown University/Twinbrook (London); Bioqual, Inc., Rockville, MD (Bradbury)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

<0.1

PROFESSIONAL:

<0.1

OTHER:

<0.1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Our laboratory is evaluating a rotavirus vaccination strategy in infants and young children that involves the use of an animal rotavirus strain of rhesus rotavirus origin in combination with three human rotavirus-rhesus rotavirus reassortant strains to form a quadrivalent vaccine. This approach relies on the induction of antibody to the rotavirus outer capsid protein VP7. It was adopted in an attempt to achieve protection against each of the four medically important VP7 rotavirus serotypes. If the desired level of protection is not achieved by this vaccine, we are considering two major alternative strategies: (i) the use of one or more cold-adapted human rotavirus mutants which would possess the human rotavirus VP4 (whereas the quadrivalent vaccine possesses the rhesus rotavirus VP4), an important outer capsid protein that also induces neutralizing antibodies and may be important in the induction of heterotypic immunity; and (ii) replacement of at least a single gene of a human rotavirus strain or of a human rotavirus x human rotavirus reassortant, that is associated with virulence with that of a bovine rotavirus strain, thus preserving the VP4 and VP7 specificities of the human rotavirus strain. It is important to identify an experimental animal that develops diarrhea following oral administration of a virulent human rotavirus strain in order to determine if either of these approaches yield viruses that are attenuated. Previously, we induced rotavirus diarrhea, and virus shedding and a serologic response to rotavirus in one of two chimpanzees that were given a virulent VP7 serotype human rotavirus strain by the alimentary route. Last year we administered a cold-adapted (26°C) human rotavirus reassortant that possesses ten genes (including VP4) from human strain Wa and one gene encoding VP7 from human strain DS-1 (a VP7 serotype 2 strain). The chimpanzee did not develop illness or shed rotavirus. Serologic study completed this reporting period indicated that this chimpanzee failed to develop an antibody response to rotavirus.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00639-02 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Epidemiology of Rotaviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Jorge Flores, M.D.

Visiting Scientist

LID, NIAID

COOPERATING UNITS (if any)

Medical University of Southern Africa (Steele); Institute of Virology, University of Pavia, Pavia, Italy (Gerna); Akita University School of Medicine, Akita, Japan (Nakagomi)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**TERMINATED 1993 - 1994**



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00640-02 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Synthetic Analogs of Rotavirus Genomic RNA Expressing a Foreign Marker Gene

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Mario Gorziglia, Ph.D. Visiting Associate LID, NIAID

Others: An-Dao Yang, M.D. Visiting Associate LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects   ☐ (b) Human tissues   ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

TERMINATED 1993 - 1994



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00641-03 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analysis of Neutralizing Epitopes on Rotavirus Outer Capsid Proteins (VP4 and VP7)

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Yasutaka Hoshino, D.V.M. Visiting Scientist LID, NIAID

Others: Mario Gorziglia, Ph.D. Visiting Scientist LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

<0.1

PROFESSIONAL:

<0.1

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

One serotype-specific and three serotype-cross-reactive neutralizing monoclonal antibodies (N-mAbs) were utilized to study the neutralization epitopes involved in the formation of antigenic sites. Single, double, or triple neutralization-resistant mutants were selected by using the N-mAbs sequentially *in vitro*. Nucleotide and deduced amino acid (aa) substitutions found on the VP7 of such mutants showed that: (i) in addition to variable regions VR-5 (aa 87-100), VR-7 (aa 141-150), VR-8 (aa 208-224), and VR-9 (aa 235-242), aa 290 and 291 in the constant region of the VP7 are involved in neutralization, and (ii) VR-5, VR-7, VR-8, VR-9, and aa 290 and 291 are functionally related to one another. In order to further analyze neutralization sites on the VP7 and to study possible synergistic or antagonistic effects among the N-mAbs employed in this study, antigenic variants were generated in the presence of three N-mAbs. Nucleotide sequence analysis of the VP7-encoding gene of the mutants revealed no synergistic or antagonistic effects among the mAbs used in the selection of neutralization escape mutants.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00642-03 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Characterization of Norwalk and Norwalk-Like Viruses (27 nm Viruses)

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Judy F. Lew, M.D. Senior Research Invest. LID, NIAID

Others: Kim Y. Green, Ph.D. Senior Staff Fellow LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Norwalk and Norwalk-like human caliciviruses are major etiologic agents of epidemic nonbacterial gastroenteritis. However, knowledge of the molecular epidemiology of these noncultivable viruses is limited. Our goal is to characterize viral genetic determinants that are responsible for the antigenic diversity and epidemiologic importance of these pathogens. Towards this goal, we accomplished the following this year: (i) sequence analysis of approximately 2,600 consecutive bases from the genome of the Hawaii virus, a prototype 27nm Norwalk-like virus that was identified in previous volunteer cross-challenge studies in our laboratory as serotypically distinct from the Norwalk virus; (ii) sequence analysis of a segment of the RNA-dependent RNA polymerase-encoding gene of two other Norwalk-like viruses obtained from elderly nursing home patients; and (iii) cloning of the gene encoding the capsid protein and production of baculovirus-expressed self-assembled virus-like particles (VLPs) from the Desert Shield virus (DS395), Toronto virus (TV24, formerly designated "minireovirus"), and Hawaii virus (HV). Furthermore, we have used these recombinant VLPs to develop diagnostic assays for the detection of these viruses and have initiated molecular epidemiologic studies. In addition, examination of the antigenic relationships among these viruses is in progress.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00643-02 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Expression of Rotavirus Outer Capsid Protein VP4 by a Baculovirus Recombinant

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Mario Gorziglia, Ph.D. Visiting Associate LID, NIAID

Others: Baoguang Li, Ph.D. Guest Researcher LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**TERMINATED 1993 - 1994**



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00684-02 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic and Serological Characterization of Equine Rotaviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Eileen Ostlund, D.V.M., Ph.D. IRTA Fellow LID, NIAID

Others: Yasutaka Hoshino, D.V.M. Visiting Scientist LID, NIAID

COOPERATING UNITS (If any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.7

PROFESSIONAL:

0.6

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Group A rotaviruses are an important cause of diarrhea of human infants and the young of many other species. Two rotavirus outer capsid proteins, VP4 and VP7, influence virulence and are independent neutralization antigens. Fourteen VP7 serotypes (G types) have been identified of which four (G3, G5, G13 and G14) have been detected in equine rotaviruses, with G3 as the predominant serotype. Eleven VP4 serotypes (P types) are recognized among group A rotaviruses but the VP4 serotypes of equine rotaviruses have not been characterized. To develop VP4 serotyping reagents, panels of reassortants were generated with human rotavirus strain DS-1[G2] and each of five equine rotavirus strains (H2[G3], FI-14[G3], H1[G5], L338[G13], and FI-23[G14]). Reassortants containing the genomic segments encoding the equine rotavirus VP4 with at least VP7 from the human rotavirus strain were used to prepare hyperimmune sera. VP4 relationships among the equine viruses were established by plaque reduction neutralization assays using the hyperimmune sera. To examine the relationships between equine rotavirus VP4s and those of other species, the hyperimmune sera were tested for neutralizing activity with representative rotaviruses of all 11 currently defined VP4 serotypes.

Cold-adaptation of rotaviruses may be associated with attenuation of virulence. In order to investigate the genetic basis for cold adaptation, propagation of equine rotavirus H2 in MA104 cells at progressively lower temperatures was achieved starting with routine culture at 37°C and progressing stepwise downward in temperature to 30°C, 28°C, and 26°C. Virus was triply plaque purified after the tenth passage at each temperature. Currently, the H2 virus is being adapted to grow at 25°C. Plaque purified viruses have been examined for growth and plaquing ability in MA104 cells at temperatures ranging from 26°C to 43°C.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00685-02 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic Studies of the Cold-Adapted Human Rotavirus Phenotype

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Eileen Ostlund, D.V.M., Ph.D. IRTA Fellow LID, NIAID

Others: Yasutaka Hoshino, D.V.M. Visiting Scientist LID, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.60

PROFESSIONAL:

0.35

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The role of specific rotavirus genes involved in conferring phenotypes characterized by either: (i) cold-adaptation (26°C), or (ii) ability to grow at 39°C (non-temperature sensitive) is being investigated. In the initial approach to this project, reassortants were generated by coinfection of AGMK or MA104 cells with cold-adapted (26°C), temperature sensitive human rotavirus Wa x P and wild type human rotavirus DS-1 (non-cold-adapted, non-temperature sensitive). Progeny virus was selected without antibody pressure at 30°C or 39°C. In later experiments, such reassortants were generated and selected at 34°C, a temperature permissive for both parental viruses. Some reassortants generated at 34°C were subjected to antibody pressure against the VP7 serotype of DS-1 during selection. Reassortants were identified by PAGE of genomic RNA and were plaque purified at the respective temperatures used to generate the reassortant. Rotavirus reassortants containing the Wa x P (ca) or DS-1 parental genes for VP6 and VP7 were also confirmed by ELISA.

Parental origin of each gene segment was assigned for each reassortant. Rotavirus gene segments from the Wa x P (ca) parental strain which were present in reassortants selected at 39°C include genes 2, 5, 6, 7, 8, 9, and 11. The presence of wild type rotavirus DS-1 genes 3, 4, 5, 6, 7, 8, 9, or 11 did not abrogate the ca phenotype of viruses selected at 30°C. Genetic reassortment at the permissive temperature of 34°C, in combination with antibody pressure yielded (Wa x P) x DS-1 reassortants with various constellations of genes from the parental viruses. Each Wa x P ca gene is represented in at least one reassortant from this group. Reassortants from this group have been triply plaque purified and selected reassortants will be tested for ca and ts phenotypes.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00707-01 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Identification of Genetic Determinants Required for the Replication of Caliciviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kim Y. Green, Ph.D. Senior Staff Fellow LID, NIAID

Others: Judy Lew, M.D. Senior Research Invest. LID, NIAID  
Stanislav Sosnovtsev, Ph.D. Visiting Fellow LID, NIAID

COOPERATING UNITS (if any)

Uniformed Services University of the Health Sciences, Bethesda, MD (Monroe, Vincent)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.7

PROFESSIONAL:

1.1

OTHER:

0.6

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Caliciviruses, represented by the prototype Norwalk virus, are the major cause of epidemic acute gastroenteritis in humans. A major obstacle to the study of these medically important viruses is our inability to establish their growth in cell culture. The molecular mechanisms responsible for the fastidious nature of these viruses are not known. The primary focus of this project is to gain a better understanding of the replication strategies of cultivatable strains of *Caliciviridae* and apply this information to the formulation of a successful strategy for the cell culture adaptation of the noncultivable viruses of this family. Feline calicivirus (FCV) was selected for an analysis of its replication *in vitro* because it grows efficiently in cultured, permissive cells. Experiments were initiated with FCV to study receptor binding, infectivity of viral RNA, mapping of gene products in the viral genome, protein processing, and the mechanisms responsible for host cell and cell type restriction in cell culture. Preliminary studies indicate that cell tropism of FCV involves functions subsequent to receptor binding, penetration and uncoating. Thus, FCV genomic RNA was infectious when transfected into a permissive cell line but infection did not occur when non-permissive cells were transfected.

Clones overlapping the entire NV genome were generated and recombinant proteins were expressed *in vitro*. The functions of these proteins will be examined as the FCV protein functions are elucidated. In addition, extensive efforts will continue in an attempt to identify a cell strain or line in which Norwalk virus will replicate.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00370-12 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pathogenesis Studies of Simian Immunodeficiency Virus (SIV) Infection of Macaques

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Vanessa Hirsch, D.V.M., D.Sc.	Visiting Scientist	LID, NIAID
Others:	Simoy Goldstein, Ph.D.	Visiting Scientist	LID, NIAID
	Barbara Campbell, Ph.D.	Staff Fellow	LID, NIAID
	William R. Elkins, D.V.M.	Senior Veterinarian	LID, NIAID
	Harold Ginsberg, M.D.	Fogarty Scholar	LID, NIAID
	Raymond Langley, Ph.D.	Visiting Associate	LID, NIAID

COOPERATING UNITS (if any)

LIR/NIAID/NIH, Bethesda, MD (Fauci, Fox); The Ohio State University (Johnson); The Henry Jackson Foundation (Zack, Lewis, Brown); Southwest Foundation for Biomedical Research (Allan); Los Alamos National Laboratory (Myers)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Immunodeficiency Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

9.5

PROFESSIONAL:

6.5

OTHER:

3.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The major emphasis of this project was the study of SIV pathogenesis in experimentally-infected macaques, utilizing strains derived from sooty mangabeys (SIVsm) and African green monkeys (SIVagm). SIVsm clones derived from an immunodeficient macaque were employed to study viral factors contributing to the induction of AIDS. A study of SIVsm clones derived from the spleen of an immunodeficient SIV-infected pig-tailed macaque identified a region analogous to the V3 loop of HIV-1 envelope to be responsible for determining tropism. Naturally-occurring variation in this region determined whether viruses infected macaque lymphocytes and/or macrophages and this tropism appeared to be linked to pathogenicity. The degree of variation within the envelope of viruses within tissues of SIV-infected macaques was compared utilizing single stranded conformational polymorphism (SSCP) and sequence analysis. Virus populations within lymphoid tissues were highly heterogeneous whereas, distinct and homogeneous populations were observed in the brain and PBMC.

A SIVagm strain (SIVagm9063) was isolated and molecularly cloned after experimental passage through a pig-tailed (PT) macaque. This isolate induced immunodeficiency in experimentally infected pig-tailed macaques but caused an asymptomatic infection of rhesus (RH) macaques and African green monkeys (AGM). An infectious, molecular clone produced a high viral load and reproduced the immunodeficiency syndrome in PT macaques. Probes for *in situ* hybridization were developed and analysis of tissues of PT macaques, RH macaques and AGM revealed significant diminution in viral load in the latter two species suggesting that disease was directly related to extent of infection.

Treatment of SIV infected monkeys with an inhibitor of TNF $\alpha$  (pentoxifylline) failed to alter the course of disease.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00686-02 LID

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Vaccine Studies of Simian Immunodeficiency Virus (SIV) Infection of Macaques

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Vanessa M. Hirsch, D.V.M., D.Sc. Visiting Scientist LID, NIAID

Others: Simoy Goldstein, PhD Visiting Scientist LID, NIAID  
 Noreen A. Hynes, M.D. Senior Investigator LID, NIAID  
 Barbara Campbell, Ph.D. Staff Fellow LID, NIAID  
 Malcolm Lawson Visiting Fellow LID, NIAID  
 William R. Elkins Senior Veterinarian LID, NIAID

COOPERATING UNITS (if any)

LVD/NIAID/NIH (Moss); GeneLabs (Fuerst); The Ohio State University (Johnson); The Henry Jackson Foundation (Zack, Lewis); Vanderbilt University (Montefiori); Bristol Myers Squibb (Haigwood); Chiron Corporation (Van Nest); Beth Israel Hospital (Letvin)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Immunodeficiency Viruses Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.5

PROFESSIONAL:

1.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The major emphasis of this work was the use of the SIV/macaque model of AIDS for the evaluation of potential vaccine strategies. The ability of whole inactivated SIV vaccine (WI-SIV) to modify subsequent disease course was assessed in a group of five immunized macaques challenged with cell-associated SIV. As predicted from previous experiments all macaques became infected; however, long-term evaluation showed that the immunized animals survived longer than the naive controls suggesting that immunization may beneficially modify the course of disease.

Recombinants of the highly attenuated modified vaccinia virus of Ankara (MVA) expressing the SIVsmH4 gag-pol and env were generated. The immunogenicity and protective efficacy of the MVA recombinant (MVA-SIV) was compared with that of a similar recombinant in a Wyeth vaccinia virus background (Wyeth-SIV). Four animals were immunized with each recombinant virus a total of five times at approximately three month intervals. Whereas, the MVA-SIV boosted SIV antibody levels following sequential inoculations, the Wyeth-SIV recombinant only induced a boost after the second immunization and antibody levels declined subsequently. Thus, MVA-SIV appeared to more immunogenic than Wyeth-SIV. Although immunization did not prevent infection following intravenous challenge with uncloned SIVsm, the virus load of three of the four MVA-SIV-immunized animals was 100- to 1,000-fold reduced compared to animals immunized with nonrecombinant vaccinia virus. Also, plasma viremia was not detectable in the MVA-SIV group. Thus, immunization with MVA-SIV resulted in significant restriction of virus replication and dissemination which may be predictive of longterm survival of these animals.



# LABORATORY OF MOLECULAR MICROBIOLOGY

## 1994 Annual Report

### Table of Contents

<u>Z01 AI Project Number</u>	<u>Title</u>	<u>Page</u>
Summary		13-1
00027-27	Basic Studies of Mycoplasmas - Tully	13-22
00190-16	The Molecular Genetics of Eukaryotic Cells and Their Viruses - Martin	13-23
00300-13	Genetic Aspects of Viral Oncogenesis in Wild Mouse Species and Laboratory Mice - Kozak	13-24
00301-13	Genetic Mapping of Mouse Chromosomal Genes - Kozak	13-25
00304-13	Pathogenesis of Retroviral Diseases - Silver	13-26
00415-10	The Molecular Biology of Retroviruses Associated with AIDS - Martin	13-27
00467-08	Biochemical & Genetic Studies of HIV RNA Packaging in the Nascent Particles - Venkatesan (terminated)	13-28
00527-07	Molecular Cloning and Characterization of Retroviruses Associated with AIDS - Theodore	13-29
00528-07	Functional Studies of HIV Regulatory Proteins - Venkatesan	13-30
00547-06	Molecular Mechanism(s) of Human Retrovirus <i>trans</i> -Regulatory Proteins - Jeang	13-31
00669-02	Molecular Analysis of Retroviral Genes and their Products - Strebel	13-32





00689-01	A Genetic & Biological Analysis of HIV-1 and HIV-2 - Peden (terminated)	13-33
00715-01	RNA-Mediated Recombination - Derr	13-34



# LABORATORY OF MOLECULAR MICROBIOLOGY

## SUMMARY

When it was originally established in 1981, the staff scientists of the Laboratory of Molecular Microbiology (LMM) investigated the structure, function and regulation of a diverse group of microorganisms including RNA and DNA viruses, aerobic and anaerobic bacteria and mycoplasmas. Currently, the principal focus of LMM scientists is murine and primate retroviruses although small programs involving mycoplasmas and mouse genetics still exist. In FY 1994, the principal area of research activity continues to be studies of the human immunodeficiency virus (HIV). Fundamental investigations of viral gene regulation, protein structure and function, and particle assembly are integrated with studies of the determinants of immunologic protection against HIV and viral pathogenesis.

## RESEARCH INVOLVING HIV

### BIOLOGY OF HIV INFECTIONS

Rapid induction of apoptosis by cell-cell transmission of HIV. The role of programmed cell death (apoptosis) in HIV-infected cells has been investigated using a synchronous cell-cell transmission model. In this system, HIV DNA synthesis is completed within 4-6 h and new extracellular virion production occurs within 16 h of cocultivation. The onset of the oligonucleosomal DNA degradation pattern characteristic of apoptosis was detected within 12 h of cocultivation. Apoptotic nuclei were present in single cells and in syncytia. The onset of apoptosis required Env-CD4 interactions, and was prevented by the presence of soluble CD4 in the medium. In contrast, induction of apoptosis did not require new HIV replication, and was unaffected by AZT, which completely prevented reverse transcription and progeny virion production. Apoptosis occurred in cocultivations arrested in S-phase, demonstrating that progression through G2/M phase of the cell cycle was not required. Apoptosis was also identified in cocultivations of chronically infected H9 cells with peripheral blood lymphocytes from HIV seronegative humans or chimpanzees, demonstrating that apoptosis was not restricted to lymphocytic cell lines or human cells. Apoptosis also occurred in PBMCs depleted of CD8<sup>+</sup> cells, suggesting that apoptosis was not due to CD8<sup>+</sup> cytotoxic T cells. (Maldarelli, Berthold and Martin)

Analysis of early events of HIV-1 replication preceding integration. Although HIV infection occurs rapidly in activated T-cells and monocyte derived macrophages, infection of quiescent CD4<sup>+</sup> lymphocytes proceeds only to reverse transcription; new HIV protein synthesis is not detected, and no extracellular virions are produced. Because the asynchronous nature of cell-free HIV infections of lymphocytes has hindered investigation of the sequence and kinetics of early events in HIV replication, used a highly synchronous cell-cell transmission system was used instead. Cocultivation of uninfected H9 cells with H9 cells chronically infected with HIV<sub>III</sub> results in synthesis of HIV DNA within 4 h, viral protein synthesis within 10 h, and HIV production after 16 h. Cocultivation of chronically infected



H9 IIIB cells with unstimulated PBLs resulted in the production of full-length copies of linear HIV DNA but no evidence of HIV protein synthesis or virus production. To investigate the role of specific HIV gene products in early replication events, a series of H9 cell lines chronically expressing molecular clones of HIV with mutations in specific genes potentially involved in transmission are being prepared. The first mutant under study being evaluated is an integrase defective derivative to characterize early events preceding integration, and specifically, to ascertain whether integration is required for new synthesis of HIV mRNA following cell-to-cell transmission. (Maldarelli, Sato, and Martin)

An infectious clone of a macrophage-tropic HIV-1 isolate. Unintegrated, circularly permuted viral DNA was cloned from the Hirt fractionated HIV<sub>AD-87m</sub> infected cellular DNA using an *EcoRI* digested phage vector DNA. One of the 9.2 kb single LTR clones obtained was inserted into pBR322 and, following transfection of HeLa cells, directed the production of virus capable of infecting human monocyte derived macrophage (MDM). This clone was converted to a two-LTR linear HIV-1 proviral DNA, designated pAD-2. Studies of the spontaneous shedding of gp120 from purified virus particles revealed that the macrophage-tropic AD8-2 virion-associated envelope is different from the gp120 present on T-cell line tropic strains of HIV-1. Mutations affecting the *vpu*, *vpr* and *nef* genes modestly reduced the infectivity of HIV<sub>AD8-2</sub> in both PBMC and MDM. (Theodore, Englund and Martin)

HIV integrase is required for productive infections of human monocyte derived macrophages. Macrophage tropic HIV-1 isolates are able to productively infect non-dividing cells of the monocyte/macrophage lineage, such as microglial cells in the central nervous system of seropositive individuals. To date, a molecular genetic approach has not been used to unequivocally demonstrate that integration of HIV-1 DNA is required to establish a productive virus infection in cultured non-divided macrophages. Toward this end, an in-frame deletion mutant of the central, catalytic domain of HIV-1 integrase was constructed in the context of an infectious macrophage tropic molecular clone of HIV-1. This mutant failed to infect PHA-stimulated peripheral blood mononuclear cells (PBMC), M-CSF treated primary monocyte-derived macrophage (MDM), and MDM differentiated in the absence of exogenously added cytokine. PCR analyses indicated that IN mutant virus entered MDM, reverse transcribed its RNA, but was unable to complete the replication cycle because of a presumed block to integration. These data establish integration as an obligate step in HIV-1 infections of activated PBMC and primary human macrophage cultures by cell free virus preparations. (Englund, Theodore and Martin)

## THE HIV LONG TERMINAL REPEAT AS A REGULATOR OF TRANSCRIPTION AND INFECTION

A replication-impaired HIV-1 reverts by altering TATA box sequences. Second-site revertants from replication-incompetent molecular clones of HIV with deletion mutations affecting the Sp1 motifs, contain base substitutions adjacent to the TATA motif. The altered TATA box motifs were analyzed for their effect(s) on virus infectivity, LTR-directed expression in transient transfection assays, *in vitro* RNA synthesis, and assembly of the TFIID/TFIIA preinitiation complex. The revertant TATA boxes accelerated the kinetics of



HIV replication when present in the context of an LTR containing a Sp1 mutation (deletion or site-specific); no effect was observed on the infectivity of wild type HIV. In CAT assays and *in vitro* transcription systems, the altered TATA box motifs led to elevated basal levels of RNA synthesis from NFkB/Sp1 mutagenized or wild-type templates, respectively, but did not increase responsiveness to Tat transactivation. The revertant TATA boxes accelerated the binding of TFIID and TFIIA to the LTR and stabilized their association with the promoter. The revertants did not assemble a more processive elongation complex. These results suggest that in the context of an impaired enhancer/promoter (*viz.* mutated Sp1 elements), a series of HIV revertants emerge which contain LTR alterations that significantly augment basal RNA synthesis. The TATA motif revertants are capable of rescuing the enhancer/promoter defect and sustain virus infectivity. (Shibata, Brady, Martin)

HIV-1 U5/LTR mutants. Non-overlapping deletions that eliminated the 5' (HIV-1<sub>U5/603del</sub>), middle (HIV-1<sub>U5/206del</sub>), and 3' (HIV-1<sub>U5/604del</sub>) thirds of the U5 region of the HIV long terminal repeat (LTR) were studied for their effects on virus replication and infectivity. All three mutants exhibited a wild type phenotype in directing the production and release of virus particles from transfected HeLa cells. In infectivity assays, HIV-1<sub>U5/206del</sub> was usually indistinguishable from wild type virus whereas HIV-1<sub>U5/603del</sub> failed to infect human PBMCs or MT4 and CEM cells. Investigations of HIV-1<sub>U5/603del</sub> particles revealed a packaging defect resulting in a 10-fold reduction of encapsidated genomic RNA. The HIV-1<sub>U5/604del</sub> mutant was either non-infectious or exhibited markedly delayed infection kinetics, depending on the cell type and multiplicity of infection. Quantitative competitive PCR indicated that HIV-1<sub>U5/604del</sub> synthesized normal amounts of viral DNA in newly infected cells. During the course of a long term infectivity assay, a revertant of the HIV-1<sub>U5/604del</sub> mutant emerged that displayed rapid infection kinetics. Nucleotide sequence analysis indicated that the original 26 nucleotide (nt) deletion present in HIV-1<sub>U5/604del</sub> had been extended an additional 19 nts in the revertant virus. Characterization of the HIV-1<sub>U5/604del</sub> mutant LTR in *in vitro* integration reactions revealed defective 3' processing and strand transfer activities that were partially restored in the revertant LTR substrate, suggesting that the reversion corrected an integration defect in the HIV-1<sub>U5/604del</sub>. (Vicenzi, Purcell, Englund and Martin)

## HIV RNA SYNTHESIS AND STABILITY

Chromatin structure and HIV-1 transcription regulation. In eucaryotic cells, DNA is organized into nucleosomes (histone octamers) and packaged into chromatin fibers. Nucleosome formation may permit or impede the access of soluble transcription factors to their cognate sites in the DNA, thereby playing an active role in transcription regulation. Independent studies have demonstrated that the highly compacted regions of chromatin in the genome are transcriptionally silent with transcription occurring in areas of "open" chromatin. To assess the effect of chromatin structure on the HIV-1 promoter, HeLa cells were stably transfected with wild-type HIV-1 LTRs linked to a CAT reporter gene. Pools of hygromycin-resistant cells were analyzed for constitutive and inducible expression of CAT activity. Our results indicated that the integrated HIV-1 LTR-promoter is markedly downregulated in HeLa cell lines and is only weakly inducible by TNF or PMA, previously shown to stimulate the unintegrated HIV-1 promoter present in transient transfection assays. The repression observed





is likely due to the nucleosomal organization of the viral promoter. In contrast, the promoter activity of the integrated HIV LTR is highly inducible following transfection of Sp-1, NFkB (p65 and p50) or Tat expression vectors. This result suggests that under physiological conditions, activation of the integrated promoter requires multiple inducers to overcome the histone repression. The chromatin structure of the LTR-promoter in independent HeLa cell clones, containing one copy of the LTR-CAT construct, is currently being evaluated prior to and following activation. (El Kharroubi and Martin)

Binding of transcription factor downstream of the HIV-1 promoter. A previous analysis of the HIV-1 provirus in chronically infected human T cell lines localized open chromatin domains downstream of the transcriptional start site within the leader sequence. This region exhibited increased accessibility to nucleases such as DNase I and was designated nuclease hypersensitive site 4 (HS 4). DNase I hypersensitive sites are often associated with the regulatory regions of cellular and viral genes. HS 4 has been mapped by *in vivo* and *in vitro* footprinting. Four sequence specific DNA binding factors recognize this region *in vitro*: AP-1, Sp-1, an AP-3 like factor, and a new constitutive cellular factor, DBF-1 (Downstream Binding Factor 1). Point mutations of the AP-1, AP-3-like and DBF1 sites that abolish binding activities, reduced reporter gene expression in transient transfection assays to 10-30 % of the wild-type basal promoter activity. To address the potential contributions of AP-1/AP-3 like sites, and the DBF1 site on HIV LTR-directed transcription, oligonucleotides containing binding sites for DBF1 or AP-1/AP-3 motifs were cloned upstream of HIV-1 TATA box (minimal promoter) linked to a CAT reporter gene. The resulting plasmid constructs are being analyzed by transient transfection assays in different human cell lines. Point mutations affecting AP-1/AP-3 and DBF1 binding sites on replication and infectivity of an HIV-1 molecular clone are currently being evaluated. (El Kharroubi, Zensen, Martin)

Characterization of cis-inhibitory sequences in HIV-1 gag mRNA. HIV-1 mRNAs are expressed from a single 5' LTR promoter as genome-length transcripts that are alternatively spliced to encode at least 10 different HIV proteins. Using a series of reporter plasmids, we and others have identified regulatory sequences within the *gag/pol*, and *env* genes which post-transcriptionally repress their own expression, and which require Rev/RRE for expression. To investigate the effects of inhibitory regions, introns, and splice sites on the expression of HIV-1 *gag* mRNA and protein, a recombinant CMV-driven *gag* plasmid, pCMVgag-2, was constructed. In transient transfection studies, two distinct mRNA species were expressed from pCMVgag-2: 1) full-length pCMVgag-2 mRNA, and 2) a short mRNA generated by splicing from the major splice donor (MSD) to a cryptic splice acceptor within the *gag* gene. Because of the presence of inhibitory sequences, however, negligible Gag protein expression was detectable. The presence of the RRE in *cis* and Rev in *trans* increased Gag expression 20-50 fold. Deletion of the HIV-1 MSD or insertion of the efficiently spliced IVS-2 intron from the rabbit  $\beta$ -globin gene upstream of the *gag* gene increased Gag protein expression 8-10 fold, even in the absence of Rev/RRE. *In situ* hybridization of *gag* sequences and immunofluorescent studies revealed that unspliced *gag* mRNA directed by pCMVgag-2 accumulated in discrete regions of the nucleus but did not colocalize with the SC-35 antigen, present in active splicing complexes, or with the coilin protein, a constituent of nuclear coiled bodies. In contrast, *gag* transcripts expressed from constructs containing the IVS-2 sequence did colocalize with the SC-35 antigen of active splicing complexes. These findings suggest



*gag* transcripts containing inhibitory sequences are not arrested within splicing complexes, but may accumulate within a distinct intranuclear compartment. (Berthold and Maldarelli)

Alternative mRNA splicing of HIV modulates viral replication and infectivity. The biological significance of the multiple and alternatively spliced mRNA species was evaluated during productive HIV-1 infections of peripheral blood lymphocytes (PBLs), human T cell lines, and monocyte-derived macrophage (MDM). Mutations of competing splice acceptor sites located in the center of HIV genomic RNA caused marked alterations in the pool of virus-derived mRNAs and, in some instances, markedly altered virus infectivity and/or the relative amounts of viral proteins. This result indicates that redundant RNA splicing signals in the HIV-1 genome and alternatively spliced mRNAs provide a mechanism for regulating the relative proportions of HIV-1 proteins such as gp160/120 or Nef and, in some cases, viral infectivity. HIV-1 strains tropic for MDM preferentially utilize splice acceptors for *rev* and *vpu/env* that are infrequently used by T-cell tropic viruses. The patterns of RNA splicing displayed by the macrophage-tropic proviral clone HIV-1<sub>AD8</sub> did not depend on the cell type in which viral RNA was processed; the same RNA profile was found during infections of MDM, PBLs, or after transfection into HeLa cells. The ratio of synthesis of gp160/120 compared to p55<sub>gag</sub> protein was higher for HIV-1<sub>AD8</sub> and other macrophage-tropic isolates than for HIV-1<sub>NL4-3</sub> and other T-cell line tropic isolates and was associated with a distinct profile of alternatively spliced mRNA transcribed by these macrophage-tropic isolates. The profile of alternatively spliced mRNA was dependent upon the sequence of the viral genome around the region of the competing splice acceptor sites located in the first coding exon of *Tat*. (Purcell, England and Martin)

Alteration of HIV-1 RNA splicing using antisense oligonucleotides spanning splice sites. HIV-1 generates a distinct pattern of spliced mRNAs which encode essential regulatory proteins needed for successful replication. Point mutations of some of the splice donor (SD)/splice acceptor (SA) sites, used to encode these regulatory proteins (SD1, SA4b, SA5, and SA7), have been shown to specifically alter the HIV-1 splicing pattern and virus infectivity. Using a semi-quantitative PCR assay to assess the specific effects of antisense oligonucleotides on splicing, one antisense oligonucleotide has been identified from several tested (SD1, SA2, SD2, SD3, SA4, SA4a, SA4b, SA5, SD5, SA7), that specifically affects the splicing of the mRNAs encoding the essential protein *Tat* (SA4). Synthesis of all four *Tat* mRNAs were reduced in a dose dependent manner. This antisense oligonucleotide is directed against the splice acceptor site used to encode all HIV-1 *tat* mRNAs and markedly inhibited virus replication. Preliminary experiments indicate that in the presence of the SA4 antisense oligonucleotide, cryptic splice acceptor sites are used to circumvent the splicing block. Other antisense oligonucleotides directed towards SD/SA sites known to affect viral replication (SA5) had little or no specific effects on splicing and only moderate if any effect on virus replication. This work has identified the inhibitory effects of an antisense oligonucleotide directed towards splice acceptor 4 for HIV-1, used for all *tat* mRNA species. (Purcell, McPhee and Martin)

Intracellular efficiencies of ribozymes targeted to HIV-1. Ribozymes targeted to sequences in U5, *pol*, *env*, RRE or R were inserted into the *nef* gene of an infectious HIV-1 proviral clone. When these proviral DNAs were introduced into HeLa CD4+ cells,



recombinant viruses containing the ribozyme tethered to genomic RNA or viral mRNAs were produced. The growth kinetics of ribozyme-containing viruses in MT4 cells were distinctly delayed when compared to control HIV. Although ribozyme sensitivity *in vitro* could be correlated with predicted secondary structures of target RNAs, a correlation with HIV infectivity could not be made. Both Zuker algorithm computer modeling of substrate RNA secondary structures and *in vitro* cleavage efficiencies cannot be reliably used to predict HIV-1 ribozyme sensitive sites *in vivo*. (Smith and Jeang)

## REGULATION OF HIV GENE ACTIVITY AND INFECTIVITY BY TAT

Interactions between Sp1 and Tat regulates HIV-1 transcription. Expression of HIV-1 is strongly activated by Tat. Tat requires three elements to function: TATAA, TAR, and upstream motifs within the HIV long terminal repeat. We have shown that the correct spatial arrangement of Tat, Sp1, and TATAA influences HIV expression. Under conditions in which basal promoter activity is unperturbed, distancing Sp1 from TATAA markedly reduced Tat transactivation. An increase in the Sp1-TATAA distance from 18 to 101 nucleotides (depending on the inserted sequence) rendered HIV-1 either partially or wholly replication defective. This critical dependence on spacing suggests that Tat-, Sp1, and TATAA-binding factors must precisely contact each other for optimal expression and replication of HIV-1. The region within Tat that binds Sp1 and vice versa has been mapped biochemically. (Chun and Jeang)

Evidence that replication and integration affects HIV-1 gene expression. Studies of integration and viral DNA replication on transcription of the HIV-1 LTR revealed that one requirement for Tat trans-activated expression differed for integrated versus unintegrated HIV-1 LTR targets. Specifically, the second coding exon of Tat, previously regarded as functionally dispensable, plays a role in transactivating integrated LTRs. The transcription profile directed by the HIV-1 LTR is also influenced by virus replication. Autonomously replicating vectors that contain the HIV-1 LTR produced patterns of transcription different from eukaryotic vectors that do not replicate. Both replicating and nonreplicating HIV-1 LTRs remained responsive to Tat. (Jeang)

A novel function of Tat in virion infectivity. Three groups of genetically altered HIV-1 proviruses were created: 1) a set of *tat* (-) viruses with a functional HTLV-I Tax inserted in *nef*; 2) a set of *tat* (-) viruses with Gal4-binding sites inserted in U3 and a Gal4-VP16 cDNA inserted in *nef*; and 3) a set of *tat* (+) HIV genomes that are 5' and 3' TAR (-) and are Gal4-binding-site (+) in U3 and Gal4-VP16 (+) in *nef*. Group (1) and (2) viruses, although *tat* (-), were fully complemented for viral gene expression based on quantitative measurements of viral protein synthesis and electron microscopy of released progeny virions. Interestingly, group (1) and (2) virions were defective in initiating spreading cytopathic infections when assayed in T-lymphocytes. Group (3) viruses, although capable of producing intact Tat protein, were not responsive to Tat for transcription/gene expression because of their TAR (-) genotype. However, this class of viral genomes produced viruses that were infectious in human PBMCs and T cell lines. All three groups of viruses are transcriptionally Tat-TAR independent. Their distinct differences in infectivity/cytopathicity provide genetic





evidence that Tat provides a transcriptionally independent function in determining infectivity and cytopathicity in the setting of a spreading viral infection. (Smith, Willey, and Jeang with Orenstein)

Contribution of the second exon of Tat to viral expression and replication. Two HIV-1 proviral molecules, both capable of only expressing the first coding exon of *tat*, were constructed. In one, the first coding exon of Tat was maintained in its usual location in the central part of the viral genome and a stop codon was introduced at residue 72. In the second construct, two stop codons were placed in *tat* and cDNAs coding for the two exons of *tat* or for only the first exon of *tat* were inserted separately into *nef*. In order to demonstrate that these plasmids express a transcriptionally active Tat protein, they were tested for their ability to transactivate the HIV LTR in co-transfection experiments. The levels of transactivation was found to be equivalent for the mutant proviral clones compared to the wild type provirus. The three mutants were then tested for infectivity in human T cells. A delay in virus production of 3 to 5 days was observed for the mutant proviral clone containing the first coding exon of *tat* in the central part of the genome and significantly longer for the mutant proviral clone containing the first coding exon of *tat* inserted into *nef*. These results suggest that the second coding exon of *tat* contributes to optimal virus replication. (Neuvet and Jeang)

Human autoantigen La is a TAR RNA-binding protein. The *in vivo* and *in vitro* binding of human autoantigen La to the HIV-1 TAR element has been characterized. In immunoprecipitation studies using anti-La serum, La-TAR RNPs were recovered from HIV-1 infected lymphocytes. Analysis of this binding interaction revealed that La has preferential recognition for the particular structural context of a U-rich sequence in the TAR stem. This structural component to La-TAR interactions was confirmed in competition studies and demonstrated that RNA polymers with higher order structures affected complex formation while neither single-stranded nor double stranded RNAs had any affect. The region in La that binds to pol III transcripts was also shown to interact with TAR using deletional analyses. (Jeang with Keene)

TAR RNA-binding protein maps to human chromosome 12 and mouse chromosome 15. TRBP is a human cellular protein that binds HIV-1 TAR RNA; its RNA binding domain has been mapped to amino acids 247-267. The gene for TRBP is located on human chromosome 12 and mouse chromosome 15. Interestingly, the TRBP gene on human chromosome 12 is coincident with a previously mapped human factor also on chromosome 12 that has been shown to facilitate Tat activation of the HIV-1 LTR. (Gatignol, Jeang, Kozak and McBride)

RNA-binding proteins are induced by DNA-damaging agents. A technique to detect RNA-binding proteins (RBP) involving the hybridization of an RNA probe to proteins immobilized on a membrane was used to study RBP in different types of mammalian cells. With this approach, up to 13 proteins of different sizes were detected in crude nuclear extracts using the HIV-1 transactivation-responsive (TAR) element as probe. The TAR RNA probe contains the stem-loop structure present in all nascent HIV-1 transcripts. A G+C-rich probe with similar structure also bound to many of these RBP. Other RNA probes lacking





this structure, bound to a 102-kDa protein while a probe with an A+U-rich stem-loop structure failed to bind most RBP, indicating an RNA secondary structure preference. The expression of RBP varied greatly in nine different human and hamster cell lines, with no detectable RBP in two human myeloid lines. The RBP were induced in six of seven lines following treatment with DNA-damaging agents; UV irradiation was the most effective. In Chinese hamster ovary cells, all five of the RBP present in untreated cells rapidly increased in activity after UV irradiation, and eight additional RBP were detected. The induction of these RBP by DNA-damaging agents indicates one or more possible roles for these proteins in the cellular response to genotoxic stress and in viral activation after such stress. (Gatignol and Jeang)

## THE STRUCTURE AND FUNCTION OF THE REV PROTEIN

Fine-structure mapping of the N-terminal domain of Rev: Derivation of four classes of non-overlapping dominant interfering mutants. It has been previously shown that the direct interaction of Rev with its cognate target, RRE RNA, is not required for Rev function: Rev activated the heterologous MS2 phage translation operator RNA if it was directed to it by the MS2 coat protein fusion, Rev/MS-C. The arginine-rich RNA binding domain of Rev could be functionally replaced by a stretch of 9 arginine residues. Poly-arginine insertions that preserved the Rev response for RRE RNA localized to the nucleoli; those with poor response accumulated mostly in the cytoplasm. Poly-arginine substitutions of residues 24 to 60 of Rev preserved activation for MS2 RNA, but not for RRE. Four non-overlapping classes of dominant interfering Rev mutants were located near the N-terminus of Rev between: i) residues 18 and 25; ii) 25 and 34; iii) 43 and 50; and iv) 51 and 60. Peptides corresponding to Rev residues between 16 and 85 or 23 and 85 or containing poly-arginine residues in place of the RNA binding and NLS motifs (35 to 50) were synthesized. The Rev peptides bound efficiently to RRE RNA and to the Rev responsive RRE sub-domain; they bound much less efficiently to TAR, MS2, and RexRE RNAs. (Nam, Gerstberger, Coligan and Venkatesan)

A cellular protein that binds HIV RRE RNA and several other structured RNAs is an inhibitor of the interferon induced PKR kinase *in vitro* and *in vivo*. A cellular protein, RBF, that binds to the HIV-1 Rev responsive element (RRE) in RNA has been cloned and characterized. RBF was identical to the previously reported HIV-1 TAR RNA binding protein (TRBP). *E. coli* expressed TRBP displayed binding affinities for a broad range of RNAs with an 11 bp GC rich RNA duplex constituting the minimal binding target. TRBP had two basic motifs with significant homology to several viral and cellular RNA binding proteins including the interferon inducible double stranded (ds) RNA activated PKR (DSI or DAI) kinase and vaccinia virus encoded E3L protein, a PKR kinase inhibitor. *In vitro*, TRBP was a potent competitive inhibitor of ds RNA activation of PKR kinase. This inhibition is probably mediated by heterodimerization of PKR and TRBP via a small ds RNA tether. TRBP also inhibited PKR kinase and eIF-2 phosphorylation *in vivo* in a transient expression system in which the translation of a reporter gene was inhibited by localized activation of PKR kinase. Under transient expression conditions in HeLa cells, TRBP complemented the E3L mutant of vaccinia virus that was defective for virus replication and protein synthesis. TRBP may represent a cellular regulatory protein(s) that binds to RNAs containing specific



secondary structure(s) and that inhibit the PKR kinase in a localized manner. (Park, Nam, Sonenberg and Venkatesan)

An expression competent Rev(-) HIV-1 as a possible vaccine virus. Replication of HIV-1 is dependent on the viral Rev protein which acts in concert with the *cis*-acting Rev-responsive element to facilitate nuclear export of intron-containing HIV RNAs. We have demonstrated that a *cis*-acting 219-nucleotide sequence from an unrelated and "simple" retrovirus, Mason-Pfizer monkey virus (MPMV), permits Rev-independent HIV-1 replication. This sequence is present in an untranslated region near the 3' end of the MPMV genome and is able to substitute for Rev for expression of Gag/Pol and Env proteins from subgenomic constructs of HIV-1. The MPMV element, like the HIV encoded Rev protein, may function by interacting with a cellular factor involved in mRNA transport. (Jeang, Rekosh and Hammariskjold)

### MUTATIONAL ANALYSIS OF HIV GAG PROTEINS

Mutational analysis of the HIV-1 matrix. To define domains and specific amino acid residues of the matrix protein (MA) that are involved in virus particle assembly, 35 individual amino acid substitution mutations were introduced into the HIV-1 MA. The mutants were assessed for their ability to form virus particles and to infect human T cells using reverse transcriptase assay and radioimmunoprecipitation analyses and transmission electron microscopy. Several domains of MA into which single amino acid substitutions dramatically reduce the efficiency of virus particle production were identified. These domains include the six amino-terminal residues of MA and the regions of MA between amino acids 55 to 59, and 84 to 95. Single amino acid substitutions in one of these domains (between MA amino acid 84 to 88) resulted in a re-direction of virus particle formation to sites within cytoplasmic vacuoles. Additional mutational analyses have focused on: (i) characterizing a highly-basic domain located near the N-terminus of MA and (ii) identifying residues in MA that are involved in the putative interaction between MA and the envelope glycoproteins. During the course of these studies, residues have been identified that appear to block early events in the HIV-1 life cycle (without affecting the levels of envelope on virus particles), and that are critical for envelope incorporation onto virus particles. (Freed and Martin)

Characterization of domains in the HIV-1 p6 Gag protein required for the virus particle production. A series of nonsense or single amino acid substitution mutations were introduced throughout the p6 Gag protein in the full-length proviral clone, pNL4-3. Truncation of p6 at residue 1, resulted in a 20-fold reduction in the release of virion-associated reverse transcriptase (RT) activity compared to wild type virus. The C-terminal region of p6 does not appear to be involved in virus particle production since neither premature termination nor single amino acid substitution mutations caused significant reduction in the release of progeny particles. Single amino acid changes located in a domain located between residues 7 to 10 (PTAP) markedly reduced particle production to a level similar to the p6 deficient mutant. In contrast, other individual amino acid substitutions within the N-terminal domain of p6 had little effect on infectivity. Mutations in PTAP delayed the establishment of a productive infection in the CEM (12D7) T-cell line while other



mutations had little effect on infectivity. These results indicate that PTAP is a critical domain within p6 Gag for virus particle production. Interestingly, the requirement of p6 for virion production is eliminated if the viral protease is inactivated. (Huang, Martin, Freed)

## STRUCTURAL AND FUNCTIONAL ANALYSES OF THE HIV ENVELOPE PROTEINS

Analysis of intra-gp120 interactions on HIV envelope function. To identify regions of the HIV-1 envelope glycoprotein that are involved in interactions necessary for proper envelope function, a series of fourteen envelope recombinants of the *env* genes of two HIV-1 isolates were constructed. The envelope chimeras were examined for their ability to induce syncytia, to be proteolytically processed, and to function during a spreading viral infection. The exchange of the first and second hypervariable regions (V1/V2) of gp120 resulted in defects in envelope glycoprotein processing, syncytium formation and infectivity. Long-term passage of cultures infected with virus bearing a V1/V2 chimeric envelope glycoprotein resulted in the emergence of a revertant virus with replication characteristics comparable to wild-type. Analysis of the revertant indicated that an Ile-> Met change in the C4 region of gp120 (between hypervariable regions V4 and V5) was responsible for the revertant phenotype. This single amino acid change restored infectivity without significantly affecting gp160 processing, CD4 binding, or the levels of virion-associated gp120. While the Ile->Met change in C4 greatly enhances the fusogenic potential of the V1/V2 chimeric envelope glycoprotein, it has a detrimental effect on syncytium formation when analyzed in the context of the wild-type envelope. These results suggest that an interaction required for proper envelope glycoprotein function occurs between the V1/V2 and C4 regions of gp120. (Freed and Martin)

Characterization of intra-envelope interactions occurring during infection of primary human macrophages. HIV-1 recombinants have been constructed involving exchanges of the *env* genes of the macrophage-tropic isolates, AD8 and SF162. Several recombinants are blocked in their ability to infect primary human macrophages despite being able to infect human PBMC. The region conferring the defect maps to a domain that includes the C2 and V3 portions of gp120. Addition of the congenic V1/V2 domain to these chimeras restores macrophage infection, raising the possibility that an intra-gp120 interaction required for macrophage infection occurs between V3 and V1/V2. We are currently defining more precisely the domains and residues involved in this interaction and characterizing the nature of the macrophage-specific defect. (Freed and Martin)

Chronic expression of HIV envelope and Vpu downregulates CD4 but does not prevent infection by HIV. In mice and chickens, inherited variants of retroviral envelope genes lead to resistance to exogenous infection with related retroviruses. To investigate a possible related system for HIV, a retroviral vector that confers stable expression of HIV *vpu*, *env*, and *rev* was constructed. Because wildtype *env* leads to syncytia formation in CD4-positive cells, *env* mutants that bind CD4 but do not cause syncytia were used instead. The modified vector led to stable expression of HIV Env in HeLa-CD4 cells and a 10-fold reduction in cell surface CD4, but unexpectedly, the decreased CD4 was detected in only a subpopulation of cells (about 40% of the total) derived from individual clones. Cells





expressing high and low amounts of CD4 could be separated by FACS analysis but each cell type gave rise to both high and low CD4-expressing cells after several days in culture, suggesting that down-regulation of CD4 depends on unrecognized physiological variables. Studies with vectors containing stop codons in *vpu* or *env* indicated that downregulation of CD4 required expression of both genes. Cell lines expressing *vpu*, *env* and *rev* were more, rather than less, susceptible to HIV, as were cell lines expressing *rev* alone, probably because enhanced expression of *rev* facilitates HIV infection in this system. (Fujita and Silver)

Structure/function studies of the gp120 V3 domain. Some isolates of HIV-1 are restricted in their ability to productively infect different CD4+ cell types. Several studies have shown these differences in tropism are controlled by specific regions within the viral envelope, especially the third variable (V3) region in gp120. A series of V3 recombinant viruses were constructed to examine virus tropism and specific physical/biological properties of the virion-associated envelope. All of the HIV *env* recombinants were able to establish a productive infection in peripheral blood mononuclear cells (PBMC) although some exhibited delayed infection kinetics compared to the parental wild type virus. Several HIV recombinants, however, were unable to infect a continuous CD4+ T-cell line permissive for the parental virus and also exhibited a marked decrease in the kinetics of virion gp120 binding to soluble CD4. Additional analyses of the virion gp120s demonstrated that most of the V3 substitutions affected the spontaneous release of gp120 from virions. These results indicate that V3-induced alterations in viral tropism are associated with changes in physical and functional properties of the virion envelope. (Willey, Theodore and Martin)

HIV tropism and envelope function. Previous work had indicated that single amino acid changes in both gp120 and gp41 enhanced the ability of the HIV-1<sub>ELI</sub> isolate to establish a productive infection in different human T cell lines. Potential alterations in both physical and functional properties of the virion-associated envelope were examined by monitoring the release of gp120 from purified particles. These analyses indicated that a single amino acid substitution in the CD4 binding site of gp120 enhanced both spontaneous and CD4-induced release of gp120 from virions while a single residue change in gp41 affected only CD4-induced shedding. Each codon change alone conferred increased growth kinetics. However, viruses with both changes were able to infect a variety of T cell lines. When the two mutations were present, the virions readily shed gp120, both spontaneously and following exposure to soluble CD4. Analysis of CD4 binding to virion-associated gp120 showed that the changes in both gp120 and gp41 contributed to increased binding. These results demonstrated that increased replication of HIV-1<sub>ELI</sub> in human CD4+ T cell lines was associated with altered physical and functional properties of the virion envelope. (Willey, Martin and Peden)

Virion envelope/antibody interactions. The envelope of HIV-1 is a primary target for antibody mediated neutralization. Antibodies directed against the V3 region of gp120 are only able to neutralize HIV-1 in a strain specific manner; a single amino acid change in V3 can allow escape from neutralization. More recently, specific monoclonal antibodies have been developed which recognize epitopes within both gp120 and gp41 that are conserved among widely divergent strains of HIV-1. Purified radiolabelled virions were prepared to evaluate the binding of these broadly neutralizing antibodies to the virion-associated envelope.





Our results indicate that the binding of neutralizing antibodies to both gp120 and gp41 induces the shedding of gp120 from virions to varying degrees. Antibody binding to conserved linear epitopes can be influenced by other regions of gp120 outside of the conserved antibody binding site, and affect the kinetics of binding, the overall amount of envelope bound and the stability of the envelope-antibody complex. (Willey and Martin)

## MECHANISTIC AND BIOCHEMICAL STUDIES OF THE HIV Vpu, Nef, AND Vpr PROTEINS

Cellular localization of the HIV-1 Vpu protein. The topology of the Vpu protein has recently been shown to be an integral membrane protein with the hydrophilic C-terminal domain of Vpu projecting into the cytoplasm (a type 1 integral membrane orientation). Vpu has two distinct effects on HIV-1 replication: Vpu increases the efficiency of particle release and decreases the half-life of the HIV-1 receptor protein, CD4. Using cell fractionation techniques, we have demonstrated that Vpu is synthesized on heavy membranes of the rough endoplasmic reticulum, and subsequently trafficks into a light membrane compartment. Immunofluorescence studies using a polyclonal rabbit anti-vpu antisera complemented these experiments and identified Vpu concentrated in perinuclear regions and in vesicles distributed throughout the cytoplasm. Colocalization studies using laser scanning confocal microscopy have identified Vpu in the intermediate compartment and on *cis*-Golgi membranes. A small amount of Vpu also appears to traffick beyond the *trans*-golgi network. These data suggest Vpu may affect such different events as virion production and CD4 degradation by trafficking through multiple cellular compartments. (Maldarelli, Martin, Strebel)

Structural and functional analysis of the HIV-1 Vpu gene product: Phosphorylation differentially regulates biological activities of Vpu. The HIV-1 specific Vpu is phosphorylated in infected cells at two serine residues by the ubiquitous casein kinase 2 (CK-2). To study the role of Vpu phosphorylation on its biological activity, a *vpu* mutant gene, lacking both phosphoacceptor sites, was introduced into the infectious molecular clone of HIV-1, pNL4-3, as well as subgenomic Vpu expression vectors. This mutation did not affect the expression level or the stability of Vpu, but had a significant effect on its biological activity in infected T cells and transfected HeLa cells. Despite the presence of comparable amounts of wild type and non-phosphorylated Vpu, decay of CD4 was observed only in the presence of phosphorylated wild type Vpu. Non-phosphorylated Vpu did not induce degradation of CD4 even if both proteins were artificially retained in the endoplasmic reticulum. In contrast, Vpu-mediated enhancement of virus secretion was only partially dependent on Vpu-phosphorylation. Furthermore, the effect on particle release was efficiently blocked when wild type Vpu was retained in the ER suggesting that the two biological functions of Vpu are independent, occur at different sites within a cell, and exhibit different sensitivity to phosphorylation. (Schubert and Strebel)

Mechanism of CD4 degradation by Vpu: Vpu responsive sequence in CD4. CD4 is an integral membrane glycoprotein which functions as the HIV receptor for infection of human host cells. We have recently demonstrated that Vpu induces rapid degradation of CD4 in the endoplasmic reticulum. Using an *in vitro* system, it was also shown that Vpu targets



specific sequences in the cytoplasmic domain of CD4 to promote its degradation. Transfer of the CD4 cytoplasmic domain to a heterologous protein (CD8) rendered the chimeric protein sensitive to Vpu-dependent degradation. In contrast, substitution of the CD8 transmembrane domain with the analogous region from CD4 failed to confer Vpu sensitivity. Finally, mutated derivatives of the CD4 protein containing the extracellular region alone or the extracellular and transmembrane regions linked to a heterologous cytoplasmic domain were not targeted by Vpu. Thus, sequences present in the cytoplasmic domain of CD4 are necessary and sufficient for sensitivity to Vpu. (Willey, Buckler-White and Strebel)

Mechanism of Vpu-mediated particle release. Vpu-mediated degradation of CD4 is highly specific and involves sequences in the cytoplasmic domains of both Vpu and CD4. HIV-2, unlike HIV-1, does not encode a *vpu* gene or a known functional homologue. Analysis of the kinetics of virus release from transfected HeLa cells indicated that HIV-2 release, in the absence of Vpu, is very efficient and comparable to that of HIV-1 in the presence of Vpu. However, when HIV-2 *gag/pol* genes were expressed in the HIV-1 backbone, particle release, in the absence of Vpu was markedly reduced and co-expression of Vpu with the HIV-1/HIV-2 chimera resulted in increased virion release. This result suggests that the effect of Vpu may not be restricted to HIV-1 but can act on other retroviruses if virus release is suboptimal. In the case of wild type HIV-2, virus secretion occurs at a high level and cannot be further enhanced by Vpu. It remains to be determined why HIV-2 virus release is reduced in the context of an HIV-1 backbone particularly since it is generally accepted that virion production occurs independent of Env or other viral accessory proteins, except Vpu. (Bour and Strebel)

HIV-1 Nef down-regulates the CD4 receptor by bi-modal mechanism. Nef was introduced into human T cell lines using a vaccinia virus vector expressing wild type CD4 or molecularly engineered variants. Vaccinia expressed Nef reduced the surface expression of pre-synthesized CD4 by enhancing its internalization in both CD4+ T cells and HeLa cell lines; this process was reversed by lysosomotropic agents. The loss of cell-surface CD4 required the cytoplasmic tail of CD4 but not motifs interacting with the PKC phosphorylation or *lck* p56 domains. Nef did not mediate the endosomal degradation of CD4 chimeras containing a generic, lysosome susceptible di-leucine motif in the cytoplasmic tail. In contrast to this effect on pre-synthesized CD4, co-expression of Nef and CD4 by dual recombinant vaccinia virus infections led to a biphasic defect in the synthesis and surface expression of CD4. A quantitative defect in the early biosynthetic rates of CD4 was observed followed by accelerated turnover. These data suggest that Nef can target and chaperon CD4 through intracellular pathways leading to its sequestration or degradation depending on cell type. Mutations that forced Nef translational initiation from the internal Met codon at position 20 severely impaired the Nef effect on CD4 while a mutation that abolished myristoylation by replacing the Gly with Ala at positions 2 and 3 had reduced effect on CD4. (Hiller, Chandrasekhar, Popov, Gratton, Sekaly and Venkatesan)

Vpr plays a role during HIV-1 infection of primary monocyte-derived macrophage. In fully differentiated monocyte derived macrophage (MDM), mutation of the HIV-1 *vpr* gene has only minor effects on the amount or rate of progeny virus production. In undifferentiated or partially differentiated MDM, however, the infectivity of HIV-1 containing a mutated *vpr*



is either not demonstrable or severely impaired. Quantitative, competitive PCR analysis of newly synthesized viral DNA indicates that wild type and *vpr* mutants of HIV are indistinguishable in partially differentiated MDM even though the latter failed to produce progeny virions. This result indicates that the *vpr* mutant enters and undergoes reverse transcription in undifferentiated MDM as efficiently as wild type virus but is blocked at a later point in the virus life cycle. Studies are underway to delineate the step(s) that require Vpr in HIV infections of undifferentiated MDM. (Englund and Martin)

## ANIMAL MODELS OF HIV-1 INFECTIONS

HIV transgenic mice expressing HIV genes in lymphoid and muscle tissues with myopathy and a susceptibility to opportunistic infection. Two independent lines of transgenic mice bearing full-length HIV-1 proviruses and expressing HIV genes were derived by pronuclear injection of mouse zygotes. The HIV proviral sequences were genetically engineered to contain the murine MuLV core enhancer in the place of the human NFkB enhancer motifs in the 5' and 3' HIV LTRs. Mice in both lines expressed HIV RNA in the eye lens, muscle, lymph nodes, spleen and thymus. HIV Gag-related polypeptides and HIV Env-related protein were detected in muscle. Mice heterozygous for the HIV transgene were smaller than their non-transgenic littermates and developed a severe wasting phenotype prior to premature death. Weanlings had a 'weepy' eye appearance and by two months of age, a significant fraction developed periorbital abscesses. *Pasteurella pneumotropica* was cultured from Harderian glands in young mice even in the absence of abscess formation. Histopathological examinations confirmed the presence of nasolacrimal duct as well as Harderian gland inflammatory infiltrates in young transgenic mice. The absence of infection in non-transgenic siblings suggested a susceptibility to opportunistic infection in these HIV proviral transgenic mice. (Dickie, Mounts, and Martin)

Development of a HIV-1/chimpanzee disease model using three human/chimpanzee tropic primary HIV-1 strains. Nearly all previous inoculations of chimpanzees with HIV-1 have utilized derivatives of the HIV-1<sub>LAMIB</sub> isolate and have resulted in very low virus loads and no disease. In an attempt to induce AIDS in chimpanzees, a chimpanzee was inoculated with a mixture of three primary HIV-1 isolates (DH012, 20, and 29) exhibiting tropism for chimpanzee PBMC. To achieve as high an initial virus dose as possible, large amounts of both cell-free virus and infected PBMC were inoculated into a single animal over a 2 week interval. Within the first few weeks, the inoculated chimpanzee developed a plasma viremia and lymphadenopathy, an infrequently reported occurrence in previously infected chimpanzees. The animal became infected with all three viruses, with HIV<sub>DH012</sub> generating the highest viral load (DH12>DH20>DH29). Following the resolution of the primary acute infection, the chimpanzee's immune system was stimulated by transfusing them with human white blood cells or administering high doses of steroids. Both regimens resulted in an increase of virus load (2 to 10 fold), although no significant change of CD4+ cell count was observed. So far (40 weeks after inoculation), AIDS-like symptoms have not been observed. (Shibata and Martin)

Molecular cloning of human/chimpanzee-tropic virus HIV-1<sub>DH12</sub>. To further





characterize the human/chimp-tropic HIV-1<sub>DH012</sub> strain, infectious molecular clones were generated. All four clones obtained were infectious and able to replicate in human PBMC, chimp PBMC, human primary macrophage and human T-cell lines (MT-4, H9, CEM, and C8166). All induced marked syncytia in these cell types (except for MT-4), an unusual property for macrophage tropic strains of HIV-1. In chimpanzee PBMC, the DH12-derived cloned viruses replicated more rapidly than other reported human/chimpanzee tropic HIV-1 isolates. Nucleotide sequence analyses revealed that HIV-1<sub>DH12</sub> is a member of the B clade of HIV-1 viruses, the most common type of North American HIV-1 isolates. (Shibata and Martin)

Superchallenge of previously HIV-1 infected, asymptomatic chimpanzees with a second HIV strain. Because of the absence of pathogenicity of HIV-1<sub>IIIB</sub> in chimpanzees, such infected chimpanzees can be viewed as having been vaccinated with attenuated virus. One naive chimpanzee and two HIV-1<sub>IIIB</sub> infected chimpanzees were challenged with HIV-1<sub>DH12</sub> (a human/chimpanzee tropic HIV-1 primary isolate). The naive chimpanzee became infected one week following a challenge with 30 TCID<sub>50</sub> (50 % tissue culture infection dose) of HIV-1<sub>DH12</sub>. One HIV<sub>IIIB</sub> infected chimpanzee did not become infected with HIV<sub>DH012</sub> after challenges of 100 and 1000 TCID<sub>50</sub> of virus. A second HIV<sub>IIIB</sub> chimpanzee was also resistant to a challenge of 1000 TCID<sub>50</sub> of HIV<sub>DH012</sub>. Interestingly, the pre-inoculation sera from the 2 HIV<sub>IIIB</sub> infected animals neutralized only HIV-1<sub>IIIB</sub> strain, not HIV<sub>DH012</sub>, suggesting that a cellular rather than a humoral immune response is playing the major role in this phenomenon. These animals are currently being challenged with higher doses of HIV-1<sub>DH12</sub> to see how effective the protection is. (Shibata and Martin)

Development of the SHIV/macaque monkey disease model. HIV-1/SIV chimeric viruses (SHIV) that carry the envelope gene of HIV-1<sub>IIIB</sub>, have previously been shown to infect cynomolgus monkeys both *in vivo* and *in vitro*. However, the virus loads in the infected monkeys are significantly lower than that observed following inoculation with SIV<sub>MAC</sub> strains and none developed immunodeficiency. In an attempt to improve this potentially useful system, the envelope region of the original SHIV virus was replaced with one derived from other HIV-1 strains (HIV-1<sub>MAL</sub> [an African isolate], HIV-1<sub>AD8</sub> [a macrophage tropic strain and HIV-1<sub>DH12</sub> [human/chimpanzee/T-cell/macrophage tropic primary isolate]. Of these, SHIV<sub>DH12</sub> replicated as efficiently as SIV<sub>MAC239</sub> in macaque PBMC. Two cynomolgus monkeys were inoculated with 1000 TCID<sub>50</sub> of SHIV<sub>DH12</sub>, and both monkeys became infected within two weeks. Interestingly, one monkey exhibited plasma viremia, extremely high levels of cell-associated virus and developed a significant reduction of CD4+ cells (less than 10 % of the pre-inoculation level). (Shibata and Martin)

## STRUCTURAL AND FUNCTIONAL STUDIES OF THE HTLV-I Tax PROTEIN

Mapping the activation domain of HTLV-I Tax. Fourteen mutants were constructed to delineate the minimal activation domain in HTLV-I Tax. In an assay using a Gal4-Tax (GalTx) fusion protein and a responsive promoter containing Gal4 consensus binding sites, it was shown that activation was "squashed" by co-expression of wild type Tax protein *trans*. When the Tax mutants were tested for "squashing" activity, many competed





effectively against GalTx. However, those containing changes in amino acids 289 to 322 failed to exhibit this activity. In particular, three mutants with changes at amino acids 289, 296, or 320 respectively, did not squelch GalTx activity. Mutants with individual changes at amino acids 3, 9, 29, 41, 273 or 337 efficiently inhibited GalTx function. In separate experiments, amino acids 289-322 of Tax, when fused to the DNA-binding domain of Gal4, conferred transactivation ability and activated a core promoter. Thus, amino acids 289-322 of HTLV-1 Tax define a region that contacts an essential transcription factor and represents a modular activation domain. (Semmes and Jeang)

Interactions between Tax and p53. Tumor suppressor p53 is known to be inactivated by various viral oncoproteins, such as SV40 large T antigen, HPV E6, and adenovirus 5 E1B. It was of interest, therefore, to investigate possible interactions between Tax and p53. Although no protein-protein association between Tax and p53 was demonstrated by either co-immunoprecipitation or p53-GST-Tax column chromatography, Tax functionally repressed p53-mediated activation of a responsive promoter. Expression of Tax also down-modulated p53 promoter activity. (Yim and Jeang)

## MECHANISMS OF MURINE RETROVIRAL RESISTANCE AND PATHOGENESIS

A mouse model of retroviral resistance due to expression of a retroviral envelope gene. Certain mouse strains are resistant to particular classes of murine retroviruses because they inherit related retroviral envelope genes. Using transgenic mice expressing an endogenous retroviral envelope (*Fv4*) as a transgene, it was shown that animals expressing high levels of *Fv4* envelope protein are resistant to Friend murine leukemia virus and that the resistance can be transferred by bone marrow transplantation. Interestingly, mice containing mixtures of genetically resistant and susceptible cells were partially resistant to infection in that they could recover from exogenous infections. This result is encouraging for prospects for gene therapy since it suggests that such treatment might be efficacious even if only a portion of bone marrow stem cells were successfully transduced. Unfortunately, bone marrow transplantation with the Friend virus system was much more effective in preventing a new infection than in treating a previously established infection. (Limjoco and Silver)

Mouse chromosomal genes affecting susceptibility to virus-induced neoplasms. A number of mouse genes control susceptibility to specific murine retroviruses and the diseases they induce. The progeny of genetic crosses segregating *Rmcf*, a gene responsible for resistance to the leukemogenic MCF subtype of virus *in vivo* and *in vitro* have been analyzed and this locus has been positioned on a high density map of mouse Chr 5. The inheritance of *Rmcf* has been followed in serial backcrosses to *M. castaneus*, a wild-derived mouse species which lacks endogenous MCF-type proviral envelope genes, to determine if *Rmcf* encodes a viral glycoprotein that mediates resistance by blocking receptors for retroviruses. In these crosses, a novel resistance gene inherited from *M. castaneus* has been identified, which maps to Chr 1. In other experiments, a molecular clone of the receptor for the amphotropic class of MuLV was used to position this gene on Chr 8. This information will be useful in defining the possible role of the amphotropic MuLV receptor gene in disease induction by MCF MuLVs. (Kozak and Lyu)



Tumor specific integration sites for retrovirus induced neoplasms. Nonacute MuLVs can transform mouse cells following activation of proto-oncogenes by insertional mutagenesis. Ongoing studies to define new proto-oncogenes by characterizing new common viral integration regions have focussed on viral integrations in the region of *Myb* on Chromosome 10. In two studies done in collaboration with P. Jolicoeur, two new regions of viral integrations have been identified and the genetic and physical relationship between *Myb* and these loci, *Ahi1* and *Mis2* have been defined. (Kozak)

Lymphoma in rhesus macaques viremic with recombinant murine amphotropic retroviruses following retrovirus-mediated gene therapy. The potential contamination of a gene therapy vector with replication competent retroviruses (RCR) poses a serious problem for its use in human therapy regimens given the potential tumorigenicity of murine leukemia virus (MuLV) strains in mice. Degenerate RT-PCR was used to characterize the transcriptionally active retroviral genes associated with T-cell lymphomas that occurred in two rhesus macaques participating in a gene therapy trial. Both animals received immune ablation followed by replacement with bone marrow derived stem cells that were treated with retroviral vector preparations contaminated with RCR. Each of the tumors contained a recombinant MuLV, designated Mo Ampho, that joined the amphotropic *env* gene of the helper packaging virus to the long terminal repeat (LTR) of the Moloney MuLV derived vector. A high proportion of viral RNA transcripts present in the lymphoma contained single, double and triple duplications of the second core enhancer element of the LTR that was likely to have conferred enhanced growth potential upon Mo Ampho in the rhesus and induced the lymphomas observed. One of the tumors also contained an MCF (mink-cell focus forming) envelope sequence, presumably derived from endogenous retroviral sequences present in the murine packaging cell line. The major portion of this MCF *env* sequence, designated MCF Mo Ampho, had recombined with the p15TM and LTR of Mo Ampho. A subgroup of the MCF envelope sequence was not linked with Mo Ampho indicating that effective co-packaging, infection and replication of endogenous sequences from the packaging cell line had occurred. Retroviral elements transmitted in this way would reduce the dose of intended vector, increase the rate of insertional mutagenesis and greatly increase the risk of generating RCRs. Some of these RCRs may not be detected in virus assays that depend on a predictable tropism for target cells. (Purcell, Broscius and Martin)

## MAPPING THE MOUSE GENOME

Genetic linkage studies in the mouse. Analysis of the progeny of two genetic crosses between laboratory mice and different taxonomic groups has been used to develop a multilocus genetic map of the mouse. These mice have now been typed for over 650 loci on 20 linkage groups. Some of the most recently mapped genes include transcription factors, growth factors, protein kinases, and opioid receptors. One gene, aggrecan (a major structural component of cartilage), is situated in a region of Chromosome 7 bearing the *cmd* (cartilage matrix deficiency) mutation. This gene contains a 7bp deletion in *cmd* mice thereby defining the genetic defect responsible for this mutant phenotype. Merosin, the major laminin in muscle, has been identified as a candidate altered protein responsible for dystrophia muscularis because of its map location near the gene for this disorder and its deficiency in



mutant mice. The gene for the interleukin 2 receptor gamma chain was mapped to the X Chr and its map position on this chromosome eliminated it as a candidate for the X linked disorder, xid. (Kozak, Adamson, Lyu and Filie)

### RNA MEDIATED TRANSPOSITION OF EUKARYOTIC GENES

RNA-mediated recombination in yeast. A model system has been developed in yeast, *Saccharomyces cerevisiae*, that detects the reverse transcription and chromosomal insertion of a yeast cellular transcript (*i.e.*, RNA-mediated recombination). The LTR-containing yeast retrotransposon, Ty, provides the source of reverse transcriptase activity and is also required for priming reverse transcription of the cellular transcript. Priming by Ty occurs via a template switch: reverse transcription is initiated on the Ty transcript and then switches onto the poly(A) tail of the yeast cellular RNA transcript. A LINE-like element, CRE, from the trypanosome, *Crithidia fasciculata* has also been tested for its ability to prime cellular transcripts. Because CRE is a non-LTR containing virus or poly(A) retrotransposon, its mechanism of priming and reverse transcription (unknown at present) must be different from Ty, and the CRE reverse transcriptase may have an affinity for poly(A) tails. Initial experiments used CRE fused to Ty sequences, replacing the Ty integrase, reverse transcriptase and RNaseH activities, but allowing Ty viral-like particles (VLPs) to be made. RNA-mediated recombination events were observed. More recently, CRE has been expressed under the control of the galactose-inducible, GAL1 promoter, with no Ty sequences attached. RNA-mediated recombination events were again detected, but at a much lower level. These results suggest that reverse transcription of yeast cellular RNA can occur outside of the Ty VLP. Future studies will be directed at determining the frequency of these events as well as comparing the mechanism of priming by CRE to that of Ty. (Derr)

### STUDIES OF PATHOGENIC MYCOPLASMAS

Pathogenicity of *Mycoplasma genitalium*. Both *M. genitalium* and *M. pneumoniae* have been detected in synovial fluid of a patient with acute respiratory disease and subsequent polyarthritis. Serologic analysis performed on the patient's serum revealed low antibody titers to both mycoplasma strains. However, significantly elevated levels of antibody to the unique adhesin protein (140 KDa) of *M. genitalium*, compared to that of *M. pneumoniae*, suggests that *M. genitalium* as might be a pathogenic agent for man. (Tully)





Z01 AI 00190-16-LMM

PI: Malcolm A. Martin

Title: The Molecular Genetics of Eukaryotic Cells and Their Viruses

Project Report:

The biological significance of the multiple and alternatively spliced viral mRNA species was evaluated during productive HIV-1 infections of peripheral blood lymphocytes (PBLs), human T cell lines, and monocyte-derived macrophage (MDM). Mutations of competing splice acceptor sites located in the center of HIV genomic RNA caused marked alterations in the pool of virus-derived mRNAs and, in some instances, markedly altered virus infectivity and/or the relative amounts of viral proteins. This result indicates that redundant RNA splicing signals in the HIV-1 genome and alternatively spliced mRNAs provide a mechanism for regulating the relative proportions of HIV-1 proteins such as gp160/120 or Nef and, in some cases, viral infectivity. HIV-1 strains tropic for MDM preferentially utilize splice acceptors for *rev* and *vpu/env* that are infrequently used by T-cell tropic viruses. The patterns of RNA splicing displayed by the macrophage-tropic proviral clone HIV-1<sub>AD8</sub> did not depend on the cell type in which viral RNA was processed; the same RNA profile was found during infections of MDM, PBLs, or after transfection into HeLa cells. The ratio of synthesis of gp160/120 compared to p55<sub>gag</sub> protein was higher for HIV-1<sub>AD8</sub> and other macrophage-tropic isolates than for HIV-1<sub>NL4-3</sub> and other T-cell line tropic isolates and was associated with a distinct profile of alternatively spliced mRNA transcribed by these macrophage-tropic isolates. The profile of alternatively spliced mRNA was dependent upon the sequence of the viral genome around the region of the competing splice acceptor sites located in the first coding exon of Tat.

The HIV-1 Gag proteins are involved in the assembly of virus particles, encapsidation of viral genomic RNA, and the release of progeny particles from infected cells. Structure/function relationships of domains situated in different regions of the 55 kDa Gag precursor protein was initially evaluated by introducing 35 individual amino acid substitution mutations into the HIV-1 gene encoding the 17 kDa matrix protein. The mutants were assessed for their ability to form virus particles and to infect human T cells, using reverse transcriptase assay, radioimmunoprecipitation analyses and transmission electron microscopy. Several domains of MA into which single amino acid substitutions dramatically reduce the efficiency of virus particle production were identified. These domains include the six amino-terminal residues of MA and the regions of MA between amino acids 55 to 59, and 84 to 95. Single amino acid substitutions in one of these domains (between MA amino acid 84 to 88) resulted in a re-direction of virus particle formation to sites within cytoplasmic vacuoles. Additional mutational analyses have focused on: (i) characterizing a highly-basic domain located near the N-terminus of MA and (ii) identifying residues in MA that are involved in the putative interaction between MA and the envelope glycoproteins. During the course of these studies, residues have been identified that appear to block early events in the HIV-1 life cycle (without affecting the levels of envelope on virus particles), and that are critical for envelope incorporation onto virus particles.





In a related group of experiments, a series of nonsense or single amino acid substitution mutations were introduced throughout the p6 Gag protein in the full-length proviral clone, pNL4-3. Truncation of p6 at residue 1, resulted in a 20-fold reduction in the release of virion-associated reverse transcriptase (RT) activity compared to wild type virus. The C-terminal region of p6 does not appear to be involved in virus particle production since neither premature termination nor single amino acid substitution mutations caused significant reduction in the release of progeny particles. Single amino acid changes located in a domain located between residues 7 to 10 (PTAP) markedly reduced particle production to a level similar to the p6 deficient mutant. In contrast, other individual amino acid substitutions within the N-terminal domain of p6 had little effect on infectivity. Mutations in PTAP delayed the establishment of a productive infection in the CEM (12D7) T-cell line while other mutations had little effect on infectivity. These results indicate that PTAP is a critical domain within p6 Gag for virus particle production. Interestingly, the requirement of p6 for virion production is eliminated if the viral protease is inactivated.

Finally, the potential contamination of a gene therapy vector with replication competent retroviruses (RCR) poses a serious problem for its use in human therapy regimens given the potential tumorigenicity of murine leukemia virus (MuLV) strains in mice. Degenerate RT-PCR was used to characterize the transcriptionally active retroviral genes associated with T-cell lymphomas that occurred in two rhesus macaques participating in a gene therapy trial. Both animals received immune ablation followed by replacement with bone marrow derived stem cells that were treated with retroviral vector preparations contaminated with RCR. Each of the tumors contained a recombinant MuLV, designated Mo Amphi, that joined the amphotropic *env* gene of the helper packaging virus to the long terminal repeat (LTR) of the Moloney MuLV derived vector. A high proportion of viral RNA transcripts present in the lymphoma contained single, double and triple duplications of the second core enhancer element of the LTR that was likely to have conferred enhanced growth potential upon Mo Amphi in the rhesus and induced the lymphomas observed. One of the tumors also contained an MCF (mink-cell focus forming) envelope sequence, presumably derived from endogenous retroviral sequences present in the murine packaging cell line. The major portion of this MCF *env* sequence, designated MCF Mo Amphi, had recombined with the p15TM and LTR of Mo Amphi. A subgroup of the MCF envelope sequence was not linked with Mo Amphi indicating that effective co-packaging, infection and replication of endogenous sequences from the packaging cell line had occurred. Retroviral elements transmitted in this way would reduce the dose of intended vector, increase the rate of insertional mutagenesis and greatly increase the risk of generating RCRs. Some of these RCRs may not be detected in virus assays that depend on a predictable tropism for target cells.

#### Bibliography:

Purcell, D.F. and Martin, M.A.: Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication and infectivity. J. Virol. 67:6365-6378, 1993.



Martin, M.A.: The molecular and biological properties of the human immunodeficiency virus, p. 863-907. *In* Stamatoyannopoulos, A.W. Neinhuis, P.W. Majerus, H. Varmus (eds.), The Molecular Basis of Blood Diseases 2nd ed. W.B. Saunders, Philadelphia, PA., 1993.

Freed, E.O. and Martin, M.A.: Evidence for a functional interaction between the V1/V2 and C4 domains of human immunodeficiency virus type 1 envelope glycoprotein gp120. J. Virol. 68:2503-2512, 1994.

Freed, E.O. and Martin, M.A.: HIV-1 infection of non-dividing cells. Nature 369:107-108, 1994.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00027-27 LMM

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fill on one line between the borders.)

Basic Studies of Mycoplasmas

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. G. Tully Head, Mycoplasma Section, LMM, NIAID

Others: None

COOPERATING UNITS (If any)

J. Baseman, Univ. Texas Health Science Ctr., San Antonio, TX; C. P. Davis, Univ. Texas Health Science Ctr, Galveston, TX; D. Talkington, Communicable Disease Center, Atlanta, GA; C. Bebear and J. Bove, Univ. Bordeaux II, Bordeaux, France.

LAB/BRANCH

Laboratory of Molecular Microbiology

SECTION

Mycoplasma Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Most current studies are centered on six Mycoplasma species of humans. Some of these isolates represent completely new and recently identified organisms in the urogenital tract of AIDS patients, while other mycoplasmas are previously known species that have apparently assumed new roles in association with human disease or are occurring in previously unknown tissue sites in man. Further evidence has been obtained for an important role of Mycoplasma genitalium in mixed infections with M. pneumoniae. Isolation of both mycoplasmas was made in our laboratory from synovial fluid of a patient with acute respiratory disease, and who subsequently developed polyarthritis in the wrist and knee joints. Immunoblots of the patient's serum against purified adhesin proteins from both mycoplasmas showed an exaggerated immune response to the adhesin protein (140 KDa) of M. genitalium, over than observed with the P1 (168 KDa) adhesin protein of M. pneumoniae. We believe the occurrence of M. genitalium in the joint fluids and the unusual immune response to the attachment protein of the organism offers further support for the role of the organism in human disease. Additional studies have been done on the molecular and genetic features of a group of new wall-free mollicutes (mycoplasmas). These organisms, primarily of insect origin, have been defined as new species in the genus Mesoplasma. The organisms lack a growth requirement for sterol, and differ significantly from other sterol-nonrequiring mollicutes (acholeplasmas). They have genome sizes smaller than acholeplasmas, use UGA as a tryptophan codon rather than as a stop signal, and possess enzyme II of the phosphotransferase sugar transport system (which is lacking in acholeplasmas). A phylogenetic analysis, based upon a comparison of 16S rRNA sequences, among the new organisms and other members of the class Mollicutes supports our taxonomic proposals.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00190-16 LMM

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Molecular Genetics of Eukaryotic Cells and Their Viruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. Martin	Chief	LMM, NIAID
Others:	P. Dickie	Visiting Associate	LMM, NIAID
	D. Purcell	Visiting Associate	LMM, NIAID
	E. Freed	Staff Fellow	LMM, NIAID
	A. El Kharroubi	Visiting Fellow	LMM, NIAID
	M. Huang	IRTA	LMM, NIAID
	D. McPhee	Visiting Scientist	LMM, NIAID

COOPERATING UNITS (if any)

D. Dimitrov DCBD, NCI  
A. Nienhuis St. Jude Children's Research Hospital, Memphis, TN

LAB/BRANCH

Laboratory of Molecular Microbiology

SECTION

Biochemical Virology

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

8

PROFESSIONAL:

7

OTHER:

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The function of the HIV-1 U5 LTR domain was examined by deleting non-overlapping one-thirds of the U5 region in the context of an infectious molecular clone of the HIV provirus. Deletion of the middle third of the HIV U5 had little effect on HIV infectivity whereas elimination of the 5' and 3' thirds rendered the resultant virus replication-incompetent by affecting encapsidation and a combination of preintegration functions (the reverse transcription and integration reactions), respectively. During the course of this work, a novel "second site" revertant was identified in which an original 26 nucleotide U5 deletion (eliminating the 3' one-third) had been extended an additional 19 nts. Characterization of the U5 deletion mutant in *in vitro* integration reactions revealed defective 3' processing and strand transfer activities that were partially restored in the revertant LTR substrate.

A semiquantitative polymerase chain reaction procedure was used to measure the relative amounts of alternatively spliced, steady-state HIV-1 *tat*, *rev*, *nef*, *env*, and *vpr* mRNAs synthesized during productive virus infections. The predominant species of *rev*, *tat*, *vpr*, and *env* mRNAs contain only coding exons whereas the major (4 of 5) *nef* mRNAs were incompletely spliced and invariably harbored non-coding exons. This study was complemented by introducing point mutations that eliminated the use of the major splice donor or multiple splice acceptors in an infectious molecular clone of HIV-1. Mutations that inactivated certain constitutive splice sites (e.g. the major splice donor) resulted in the activation of previously unrecognized cryptic splice sites, some of which preserved biological function. Other mutations that affected "competing" splice sites (e.g. the acceptor for the first coding exon of *Rev*) caused alterations in the populations of viral mRNAs, and in some cases resulted in loss of infectivity. These splice site mutants provide a means for assessing the functional significance of the large redundant pool of spliced HIV RNAs and the use of alternative splicing pathways for regulating HIV expression.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00300-13 LMM

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic aspects of viral oncogenesis in wild mouse species

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C.A. Kozak Res Microbiologist, LMM, NIAID

Others: M.S.Lyu LMM, NIAID

COOPERATING UNITS (if any)

P. Jolicoeur Research Inst of Montreal

LAB/BRANCH

Laboratory of Molecular Microbiology

SECTION

Viral Biology

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.5

PROFESSIONAL:

1.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A number of mouse chromosomal genes have been described which affect the susceptibility of mice to retrovirus-induced neoplastic disease. These genes include endogenous retroviral sequences, mouse cellular genes which facilitate or restrict virus replication, and proto-oncogenes disrupted by viral insertion. In the process of characterizing new common viral insertions in tumors induced by Abelson and Moloney MuLVs, we identified two new integration sites on the same chromosome, Chromosome 10. We defined the close genetic and physical distance between these insertion sites and the previously described oncogene *Myb* on this Chromosome, but suggest that these insertions may disrupt other oncogenic sequences in this region. In other experiments, we began analysis of several crosses involving DBA/2J mice which carries a locus responsible for resistance to MCF subtype of viruses, *Rmcf*. We defined the genetic map location of *Rmcf*, we identified an additional resistance gene in these same crosses which maps to Chr 1, and we are following inheritance of *Rmcf* through serial backcrosses to *M. castaneus* which lacks endogenous MCF viruses. These experiments should help determine whether *Rmcf* represents an endogenous provirus expression of which blocks viral cell surface receptors. Finally, we used a fragment of the recently clone amphotropic viral cell surface receptor to map the mouse gene to Chromosome 8.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00301-13 LMM

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic Mapping of Mouse Chromosomal Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C.A. Kozak Res. Microbiologist

Others: C. Adamson, LMM, NIAID; J. D. Filie, LMM, NIAID;

H. Chin, NINDS; M. Young, NIDR; B. Mock, NCI; K. Ozato, NICHD;

W. Leonard, NHLBI; N. Colburn, NCI-FCRC;

K. Yamada, NIDR; Y. Yamada, NIDR; S. Holland, LHD, NIAID

COOPERATING UNITS (if any)

D. Farber, UCLA; L. Gudas, Cornell; W. Nierman, ATCC; D. Stephenson, UC London;

D. Tollefsen, Washington U; D. Nebert, U Cincinnati; T. Sato, Roche Inst Mol

Biol; M. Prystowsky, Univ PA; N. Hecht, Tufts

LAB/BRANCH

Laboratory of Molecular Microbiology

SECTION

Viral Biology

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.5

PROFESSIONAL:

1.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have been involved in an ongoing effort to develop a multilocus genetic map of the mouse. Most of this work has been accomplished by the analysis of the progeny of two genetic crosses, an interspecies backcross and an intersubspecies backcross. DNAs from these mice have been typed for a variety of reference loci to permit mapping of newly defined genes of unknown map location to specific positions on the linkage map. These studies have resulted in the genetic mapping of a large number of genes including, most recently, various genes expressed in nervous tissue, transcriptional regulators, protein kinases and factors involved in bone formation. Specific map locations can be useful information since proximity to a known developmental mutation can identify such a gene as a potential candidate for the abnormal phenotype. Thus, one gene encoding a major structural component of cartilage was mapped near the *cmd* (cartilage matrix deficiency) mutation, and *cmd* mice were shown to have a 7 bp deletion of this gene. In another example, the murine merosin gene was mapped near *dy* (dystrophia muscularis) and we identified a deficiency of merosin in mutant mice suggesting that a defect in this laminin gene is responsible for this disorder. Finally, the murine interleukin 2 gamma receptor chain gene (*Il2rg*) was mapped to a position on the X chromosome near the *xid* immunodeficiency mutation, although further investigation indicated that *Il2rg* was not responsible for *xid*.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00304-13 LMM

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pathogenesis of Retroviral Diseases

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Silver	Senior Investigator	LMM, NIAID
Others:	K. Fujita	Visiting Associate	LMM, NIAID
	I. Lebedeva	Visiting Fellow	LMM, NIAID
	A. Nihrane	Visiting Fellow	LMM, NIAID

COOPERATING UNITS (if any)

none

LAB/BRANCH

LMM

SECTION

Viral Biology

INSTITUTE AND LOCATION

NIAID, 4/338

TOTAL STAFF YEARS:

3 1/2

PROFESSIONAL:

3 1/2

OTHER:

none

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We investigated the nature of resistance to Friend murine leukemia virus (F-MuLV) in transgenic mice carrying the Fv4 env transgene and worked on a related system for HIV. Transgenic mice expressing high levels of the Fv4 env transgene were completely resistant to infection with F-MuLV while mice expressing low levels of the transgene had enhanced ability to recover from infection. Resistance was transferrable from transgenic mice via bone marrow transplantation. Bone marrow chimeras containing a mixture of transgenic and non-transgenic bone marrow-derived cells were slightly resistant in that they had enhanced ability to recover from infection. Whereas bone marrow transplantation was very effective prophylactically in preventing infection, it was not very effective in treating an already established infection. We are attempting to determine whether the Fv4 gene alters the immune response to the antigenically-related F-MuLV, and are investigating alternative ways of introducing the Fv4 gene into mice as gene therapy. To build an HIV system comparable to Fv4, we made a neo-containing retroviral vector that confers stable expression of HIV vpu, env and rev after infection and selection with G418. Mutant (syncytia-negative) env genes were substituted into this vector to generate HeLa-CD4 cell clones that stably express envelope. This vector was found to down-regulate CD4 about 10-fold but only in a portion (about 40%) of cells derived from single clones. The reason for the inhomogeneity of down-regulation of CD4 is not understood but it is apparently not related to phase of the cell cycle. Clones expressing env were more, rather than less, susceptible to HIV, probably due to co-expression of rev, since clones expressing rev alone were also more susceptible to HIV. We are investigating the effects of this HIV vector system in CD4-positive lymphocytes and attempting to further down-regulate CD4 in order to block infection with HIV.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

AI 00415-10 LMM

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Molecular Biology of Retroviruses Associated with AIDS

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. A. Martin	Chief	LMM, NIAID
Others:	F. Maldarelli	Med. Staff Fellow	LMM, NIAID
	R. Shibata	Visiting Associate	LMM, NIAID
	E. Berthold	Visiting Fellow	LMM, NIAID
	M. Cho	IRTA	LMM, NIAID
	R. Zensen	Visiting Fellow	LMM, NIAID
	R. Willey	Biologist	LMM, NIAID
	G. Englund	Biologist	LMM, NIAID

COOPERATING UNITS (if any)

W. Satterfield, University of Texas, L. Arthur, Frederick Cancer Research Facility, C. Lane, NIAID, NIH, A. Schultz, NIAID, NIH

LAB/BRANCH

Laboratory of Molecular Microbiology

SECTION

Biochemical Virology

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

8

PROFESSIONAL:

6

OTHER:

2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The potential feasibility of an attenuated vaccine approach for HIV-1 was evaluated by challenging previously HIV-1 infected chimpanzees with a different strain of virus and monitoring whether "superchallenge resistance" occurred thereby preventing establishment of a second virus infection. Two chimpanzees, previously exposed to HIV-1<sub>IIIB</sub> 3 and 7 years ago, have been inoculated with escalating doses of HIV-1<sub>DH12</sub> since September 1993. In May 1993, for example, one animal received 10,000 TCID<sub>50</sub> units of virus and the other 10 ml of blood from a previously HIV-1<sub>DH12</sub> infected chimpanzee. Both animals remain protected from infection by HIV-1<sub>DH12</sub> as monitored by virus isolation and PCR.

Mutations affecting both conserved and variable domains of gp120 have been studied. An amino acid substitution that eliminated a highly conserved glycosylation site in the C2 (second conserved domain) region of gp120 had no effect on synthesis, processing or release of gp120 but drastically reduced virus infectivity. Long-term propagation of this C2 mutant gave rise to a "second-site" (in C1 or V3) revertant viruses that exhibited wild type infection kinetics. Biochemical analyses of the C2 mutant, the revertant, and wild type virus particles revealed that the mutant particles contained no gp120; this "association" defect, affecting the non-covalent linkage between gp120 and gp41, was restored by the revertant changes. This result suggested that the reciprocal interaction of C1, C2 and V3 are critical for the retention of gp120 on progeny virions. The unique properties of V3 domains derived from macrophage tropic HIV-1 isolates (conferring tropism for monocyte derived macrophages [MDM], PBLs but not for T cell lines) were studied by replacing this region of a prototype T-cell tropic cloned provirus (NL4-3) with several V3 segments from MDM-tropic HIVs. The resultant viruses lost the capacity to infect T-cells and the virion-associated envelope protein acquired altered physical and functional properties: marked reduction in the spontaneous release of gp120; lower amounts of virion associated gp120 (per particle) than the parental HIV<sub>NL4-3</sub>; and impaired binding of virions to CD4.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00467-08 LMM

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biochemical & Genetic Studies of HIV RNA Packaging inthe Nascent Particles

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. Venkatesan Medical Officer LMM, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Microbiology

SECTION

Biochemical Virology Section

INSTITUTE AND LOCATION

NIH, NIAID, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**PROJECT TERMINATED**



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 A1 00527-07 LMM

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Molecular Cloning and Characterization of Human Retroviruses**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	T. S. Theodore	Res. Microbiologist	LMM, NIAID
Others:	G. Englund	Biologist	LMM, NIAID
	M. Martin	Chief	LMM, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

**Laboratory of Molecular Microbiology**

SECTION

**Biochemical Virology**

INSTITUTE AND LOCATION

**NIAID, NIH, Bethesda, MD 20892**

TOTAL STAFF YEARS:

1.2

PROFESSIONAL:

1.0

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The genomic size of Human Immunodeficiency Virus (HIV-1) is approximately 9.7 kB with open reading frames encoding several viral proteins. Gene products (structural, regulatory and accessory viral proteins) arise from spliced and unspliced mRNAs and can affect viral replication in various cell types. Since heterogeneity among HIV-1 retroviral genomes is a distinct feature of Lentiviruses, molecular clones could provide useful reagents for studying their biochemical and physical properties.

HIV-1 isolates can be divided into two major subgroups on the basis of their cellular host range in vitro: macrophage (MT) and T-cell line tropic. MT-tropic isolates infect both macrophages and peripheral blood mononuclear cells (PBMC) but are unable to replicate in transformed CD4+ T-cell lines. T-cell line tropic isolates infect both PBMC and CD4+ T-cell lines but replicate poorly or not at all primary macrophages (MDM). Although T-cells are the major target for HIV-1 replication in peripheral blood, macrophages represent the predominant HIV-1 infected cell type in most tissues. Macrophages are probably the primary reservoir of HIV-1 and may be important for sustaining a persistent infection in individuals for many years. Most HIV-1 isolates we have cloned are T-cell tropic. We have succeeded in obtaining a complete molecular clone from a macrophage-tropic viral isolate. Preliminary biochemical and physical analyses have shown that the spontaneous shedding of the envelope protein(gp120) is drastically different from the typical T-cell line variants. Also the effects of several accessory genes (non-essential in in vitro infections) appear to be dispensable. Mutations in VPU, VPR, or NEF modestly reduced viral replication of the AD8-2 clone in either PBMC or macrophages.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00528-07 LMM

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Functional studies of HIV-1 regulatory Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. Venkatesan	Medical Officer	LMM, NIAID
Others:	S. Chandrasekhar	Fogarty Fellow	LMM, NIAID
	Y. S. Nam	Fogarty Fellow	LMM, NIAID
	S. Popov	Visiting Associate	LMM, NIAID

COOPERATING UNITS (if any)

S. Gratton	Ph.D Student	IRCM, Montreal, Canada
R. P. Sekaly	Lab Head	IRCM, Montreal, Canada
N. Sonenberg	Professor of Biochem	McGill Univ., Montreal, Canada

LAB/BRANCH

Laboratory of Molecular Microbiology

SECTION

Biochemical Virology

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

5

PROFESSIONAL:

5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In the life cycle of HIV, the Rev protein regulates the temporal switch from the early regulatory to the late lytic phase. The Rev regulatory protein of HIV is a basic nuclear protein that concentrates in the nucleoli and activates the viral RNAs, by binding to a highly structured RRE (Rev Responsive Element) RNA. Our studies of Rev and RRE RNA were targeted i) to explore the roles of RRE other than Rev binding in the activation process, ii) to analyze the various functional motifs of the Rev protein; and iii) to identify and characterize the function of the putative cellular factors that may bind RRE RNA, Rev, or both.

We have completed an exhaustive analysis of mutants in the various functional motifs of Rev both in the context of Rev and of Rev/MS2 phage coat protein chimera. The latter chimera allowed us to dissociate RNA binding from activation, and thus evaluate the roles of RRE RNA in the biochemical aspects of Rev trans-activation. From these genetic studies, we propose that poly-arginine insertion near the N-terminus of Rev promotes nucleolar targeting in a context sensitive manner, and the Rev sequence immediately flanking the 9 arginines (residues 24-35, and residues 36-50) is required for RRE RNA binding.

We have further characterized the biochemical properties of HIV-1 Rev Responsive Element (RRE) RNA binding cellular factor (RBF) that was identical to the HIV TAR RNA binding protein (TRBP). TRBP bore significant sequence homology with many cellular and viral ds RNA binding proteins including the interferon inducible ds RNA activated protein kinase, PKR. TRBP was a competitive inhibitor of ds RNA activation of interferon induced PKR kinase in vitro and in vivo. PKR inhibition may be mediated by heterodimerization between PKR and TRBP and may require a small ds RNA. We have completed our analysis of the gene structure transcriptional map of TRBP and defined its sub-cellular location.

We have extended our studies on the effect of HIV-1 NEF protein on T lymphocyte CD4 receptor. At low levels of Nef expression, the effect on cell surface expression was profound; increasing amounts of Nef resulted in a dose-dependent reduction in the steady-state levels of CD that approached 90%.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00547-06 LMM

**PERIOD COVERED**

October 1, 1993 to September 30, 1994

**TITLE OF PROJECT** (80 characters or less. Title must fit on one line between the borders.)

Molecular Mechanism(s) of Human Retrovirus trans-Regulatory Proteins

**PRINCIPAL INVESTIGATOR** (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: K-T. Jeang Medical Officer, Research LMM, NIAID  
 Others: A. Gagnol, Visiting Associate; O. J. Semmes, Staff Fellow; S. Smith, Medical Staff Fellow; C. Neuve, Visiting Fellow; R. Chun, Staff Fellow; K. Yim, Guest Researcher; J. Tal, Guest Researcher; D. Trinh, Special Volunteer; C. Zozak, Research Microb.; R Willey, Biologist, LMM, NIAID

**COOPERATING UNITS** (if any)

Drs. Rekosh and Hammarskjold, Thaler AIDS Institute, VA; Al Fornace, NCI; Jan Orenstein, George Washington U.; Jack Keene, Duke University; Wes McBride, NCI;

**LAB/BRANCH**

Laboratory of Molecular Microbiology

**SECTION**

Molecular Virology

**INSTITUTE AND LOCATION**

NIAID, NIH, Bethesda, MD 20893

**TOTAL STAFF YEARS:**

9

**PROFESSIONAL:**

8

**OTHER:**

1 (student)

**CHECK APPROPRIATE BOX(ES)**

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

**SUMMARY OF WORK** (Use standard unredacted type. Do not exceed the space provided.)

In the past year, the scientific program in the newly organized Molecular Virology Section of LMM has focused on three broad areas. These encompass: I) Molecular Regulation of HIV-1; II) Molecular Regulation of HTLV-I; and III) Molecular applications relevant for the development of HIV-1 specific ribozymes and transdominant/attenuated human immunodeficiency viruses. Here in a short format, it is not possible to present comprehensively our accomplishments over the past year. Details of our research can be better appreciated from the annual bibliography. However, we can capsule our progress in 1993-1994 with the following 11 points.

Selected findings from our research program include: 1) Understanding crucial interactions between Spl and HIV-1 Tat; 2) Obtaining evidence that HIV-1 expression is influenced by integration and replication; 3) Demonstrating that the second exon of Tat contributes to optimal expression from the viral LTR; 4) Providing evidence that Tat has a novel function in viral infectivity; 5) Elucidating the activation of cellular RNA-binding proteins in response to DNA-damaging events; 6) Identifying the human autoantigen La as a novel TAR RNA-binding protein; 7) Mapping the expressed gene of TAR RNA-binding protein (TRBP) to human chromosome 12 and mouse chromosome 15; 8) Delineating the minimal activation domain in the HTLV-I transactivator, Tax; 9) Characterizing interactions between Tax and tumor suppressor p53; 10) Constructing expression competent Rev(-) HIV-1 for use as a possible live-attenuated vaccine; and 11) Quantitating the intracellular efficiencies of five ribozymes targeted to separate loci in the HIV-1 genome.

Additional examples of studies that are ongoing and have yet reached "critical mass" include 1) developing through mutagenesis a minimally active ribozyme for HIV-1; 2) studying the roles of PKR in suppressing HIV-1 mRNA translation; 3) creating HIV-1 infectious genomes that could be treated with ganciclovir; 4) selecting for novel cellular factors that binds HIV-1 regulatory proteins using the yeast two-hybrid system; 5) engineering human parvovirus Rep gene product as a dominant suppressor of HIV-1 replication; 6) analyzing cellular genes that are induced by Tat using differential display technology; 7) selecting in vivo for more efficient Tat gene products.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00669-02 LMM

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Analysis of Retroviral Genes and their Products

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. Strebel	Senior Staff Fellow	LMM, NIAID
Others:	U. Schubert	Guest Researcher	LMM, NIAID
	S. Bour	Visting Fellow	LMM, NIAID
	M. Karczewski	Pre-IRTA	LMM, NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Microbiology

SECTION

Biochemical Virology

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

HIV is a complex retrovirus containing a number of genes not commonly found in other retroviruses. One of these genes, vpu, which is encoded only by HIV-1 and does not have any known homologues in HIV-2 or SIV, encodes for a small integral membrane protein. This protein consists of a hydrophobic N-terminal transmembrane (TM) domain and a C-terminal cytoplasmic domain. Vpu is phosphorylated by casein kinase II at two highly conserved serine residues. We have in the past identified two biological activities for Vpu: (i) enhancement of particle release; (ii) degradation of CD4. To study the function of Vpu, we constructed a series of mutants and analyzed their effect both with regard to Vpu-mediated enhancement of particle release as well as CD4 degradation. We found that phosphorylation of Vpu was absolutely essential for CD4 degradation while unphosphorylated Vpu still retained at least 50% of wild type activity with regard to particle release. Phosphorylation of Vpu did not affect stability or intracellular distribution of Vpu. Thus, the two biological functions of Vpu are differentially regulated by phosphorylation. To study the importance of the Vpu TM domain for its function, we created a mutant containing a scrambled TM domain, Vpu-ran. This protein was synthesized and integrated into cellular membranes at levels similar to wild type Vpu. As with the Vpu phosphorylation mutant, the effect of the TM mutation was assessed in the two available biological assays. In contrast to the Vpu phosphorylation mutant, Vpu-ran was still capable of inducing CD4 degradation although at a slightly reduced rate. However, this mutant was no longer able to support virus release from the cells. It also became apparent that Vpu functions at different sites in the cell: CD4 degradation necessitates the presence of Vpu in the endoplasmic reticulum (ER) while enhancement of virus release requires the transport of Vpu from the ER to an as yet unknown cellular compartment. Consequently, retention of Vpu in the ER results in enhanced CD4 degradation while at the same time obliterating the effect of Vpu on virion release. In summary, our data suggest that Vpu has two biological functions that are executed in different cellular compartments, rely on different functional domains of Vpu and may thus be mechanistically independent.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00669-02 LMM

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Analysis of Retroviral Genes and their Products

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. Strebel	Senior Staff Fellow	LMM, NIAID
Others:	U. Schubert	Guest Researcher	LMM, NIAID
	S. Bour	Visting Fellow	LMM, NIAID
	M. Karczewski	Pre-IRTA	LMM, NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Microbiology

SECTION

Biochemical Virology

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

HIV is a complex retrovirus containing a number of genes not commonly found in other retroviruses. One of these genes, vpu, which is encoded only by HIV-1 and does not have any known homologues in HIV-2 or SIV, encodes for a small integral membrane protein. This protein consists of a hydrophobic N-terminal transmembrane (TM) domain and a C-terminal cytoplasmic domain. Vpu is phosphorylated by casein kinase II at two highly conserved serine residues. We have in the past identified two biological activities for Vpu: (i) enhancement of particle release; (ii) degradation of CD4. To study the function of Vpu, we constructed a series of mutants and analyzed their effect both with regard to Vpu-mediated enhancement of particle release as well as CD4 degradation. We found that phosphorylation of Vpu was absolutely essential for CD4 degradation while unphosphorylated Vpu still retained at least 50% of wild type activity with regard to particle release. Phosphorylation of Vpu did not affect stability or intracellular distribution of Vpu. Thus, the two biological functions of Vpu are differentially regulated by phosphorylation. To study the importance of the Vpu TM domain for its function, we created a mutant containing a scrambled TM domain, Vpu-ran. This protein was synthesized and integrated into cellular membranes at levels similar to wild type Vpu. As with the Vpu phosphorylation mutant, the effect of the TM mutation was assessed in the two available biological assays. In contrast to the Vpu phosphorylation mutant, Vpu-ran was still capable of inducing CD4 degradation although at a slightly reduced rate. However, this mutant was no longer able to support virus release from the cells. It also became apparent that Vpu functions at different sites in the cell: CD4 degradation necessitates the presence of Vpu in the endoplasmic reticulum (ER) while enhancement of virus release requires the transport of Vpu from the ER to an as yet unknown cellular compartment. Consequently, retention of Vpu in the ER results in enhanced CD4 degradation while at the same time obliterating the effect of Vpu on virion release. In summary, our data suggest that Vpu has two biological functions that are executed in different cellular compartments, rely on different functional domains of Vpu and may thus be mechanistically independent.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZOI AI 00689-01 LMM

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

A genetic and biological analysis of HIV-1 and HIV-2

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: K. Peden Visiting Scientist LMM, NIAID

COOPERATING UNITS (If any)

LAB/BRANCH

Laboratory of Molecular Microbiology

SECTION

Biochemical Virology

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects   ☐ (b) Human tissues   ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

PROJECT TERMINATED



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00715-01 LMM

PERIOD COVERED

January 10, 1994 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

RNA-mediated Recombination

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. K. Derr Senior Staff Fellow LMM, NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Microbiology

SECTION

Biochemical Virology

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

RNA-mediated recombination was thought to exist because of the observation of intron loss and the presence of pseudogenes. Until recently, such processes occurring on an evolutionary time frame have been difficult to identify and characterize. An assay has been developed that allows the detection of RNA-mediated recombination, both homologous, RNA-mediated gene conversion (resulting in intron loss) and nonhomologous, RNA-mediated recombination (resulting in pseudogene formation) in yeast *Saccharomyces cerevisiae*. RNA-mediated recombination in its broadest sense requires transcription, reverse transcription and recombination. We have shown that the reverse transcriptase activity for these events is provided by the retrotransposon Ty. Structural analysis of the cellular cDNA, inserted into yeast chromosomal DNA in the absence of homology, revealed that the 5' end corresponded to the RNA start site and that the 3' end was polyadenylated. Furthermore, these cDNAs were embedded in Ty sequences. This structure suggests that Ty may also be required for priming reverse transcription of the cellular transcript and for insertion of the sequences into the chromosome. LTR-containing retrotransposons such as Ty have cis-acting sequences that specify priming and their programmed reverse transcription. However, a cellular transcript lacking such cis-acting sequences can still be reverse transcribed. We have shown that Ty is required for priming and that priming occurs via a template switch: reverse transcription is initiated on the Ty transcript and then switches onto the poly(A) tail of the cellular transcript. An alternative source of reverse transcriptase activity in mammalian cells is LINE elements or poly(A) retrotransposons. Because these elements do not contain LTRs, the mechanism of priming and reverse transcription must be different than Ty or mammalian retroviruses. Experiments in which cellular transcripts are primed, using the reverse transcriptase from a LINE-like element are being compared to those primed by the Ty reverse transcriptase. Future studies will be directed at further dissecting the mechanism of RNA-mediated recombination and identifying the viral and cellular functions required.





LABORATORY OF PARASITIC DISEASES  
1994 Annual Report  
Table of Contents

<u>Z01 AI</u>		
<u>Project Number</u>		<u>Page</u>
Summary		17-1
00094-35	<i>Entamoeba histolytica</i> : Cellular Physiology and Molecular Diagnostics - Diamond	17-11
00099-24	Biophysical Parasitology - Dvorak	17-12
00102-20	Pathogenesis of Disease Caused by Infection with Intracellular Parasites - Neva	17-13
00161-17	Immunochemistry of Parasitic Diseases - Nash	17-14
00162-18	Biochemical Cytology of Host-Parasite Interactions in Parasitic Protozoa - Dwyer	17-15
00197-15	Immunoregulation and Immune Recognition in Filariasis and Non- Filarial Diseases - Nutman	17-16
00246-12	Molecular Studies of the Genome and Surface of <i>Schistosoma mansoni</i> - Sher - Terminated	17-17
00251-13	Immunologic Studies on Schistosomiasis - Sher	17-18
00253-13	Studies of the Immunological Responses to Filarial Infections - Ottesen	17-19
00256-13	Developmental Biology of Leishmania Promastigotes - Sacks	17-20
00257-13	Immunology of Strongyloidiasis - Neva	17-21
00347-12	Studies on Schistosomal Hepatic Fibrosis - Cheever	17-22
00350-12	DNA Analysis of Parasites - Nash	17-23
00439-10	Clinical and Therapeutic Studies in Human Filariasis - Ottesen	17-24
00487-08	Studies on the Quantitative Parasitology of Schistosome Infections - Cheever	17-25
00494-08	Analysis of T Cell Responses in Human Leishmaniasis - Sacks	17-26



00512-07	Molecular Definition of Filarial Antigens - Nutman	17-27
00579-05	Studies on Immune Regulation in Toxoplasmosis and Other Opportunistic Infections - Sher	17-28



Laboratory of Parasitic Diseases  
National Institute of Allergy and Infectious Diseases  
Summary - October 1, 1993 - September 30, 1994

ADMINISTRATIVE AND ORGANIZATIONAL EVENTS

The rumble of administrative and organizational changes involving Intramural research on parasitic diseases, both the Laboratory of Parasitic Diseases (LPD) and the recently created Laboratory of Malaria Research (LMR), continues. It now appears that the two Labs will shortly be merged, as they were originally, and probably under the old name, LPD. Various factors, including the likelihood of level or reduced future Intramural funding, played a part in the decision to bring the two labs together again. The merger will also entail a change in leadership for LPD, with Dr. Louis Miller as Lab Chief, and Dr. Neva continuing as a senior investigator to pursue his clinical and laboratory research projects. Dr. Neva will also continue to serve as the Intramural representative for the NIAID Intramural Center for Tropical Diseases, which links the NIAID Extramural and Intramural supported activities in parasitic and tropical diseases as the ICTDR program (International Centers for Tropical Disease Research). The ICTDR group held its third annual meeting during April 27-29, 1994; the event gathering progressive interest and support. Drs. K. Creedon (S-adenosyl-homocysteine hydrolase as a target for anti-malarial action), C. Plowe (molecular epidemiology of anti-folate resistant malaria), and G. Lanzaro (molecular population genetics of A. gambiae), all from the malaria group, made presentations on behalf of the Intramural Labs.

Another organizational change which has had a significant impact on LPD was the decision of Dr. John Gallin, Director of the NIAID Intramural Research Program for the last 8 years, to take the position of NIH Clinical Director. This resulted in Dr. Neva taking over Dr. Gallin's position as Acting Director, DIR, until a new DIR Director is recruited. Meanwhile, Dr. Cheever as Assistant Lab Chief is helping to run the LPD.

The past year also marked the loss of several LPD Section Heads, one by retirement and the other by change in assignment. Dr. Louis "Buddy" Diamond, known world-wide for his contributions to amebiasis research, retired after 36 years of government service. His advice and expertise will still be available to LPD with his appointment as Scientist Emeritus. Dr. Eric Ottesen, who developed a "world-class" human filariasis research program in his Section of Clinical Parasitology, has accepted a temporary assignment with the World Health Organization in Geneva, which is likely to become a permanent position.

An active traffic in research fellows to and from LPD occurred in the past year. Joining Dr. Sher's group were D. Jankovic, Ph.D. (originally from Yugoslavia via Pasteur Institute in France); S. Ahmed-Fouad, Ph.D. from Egypt; and T. Sharton, a recent Ph.D. from Univ. of Pennsylvania. New additions to the Clinical Parasitology Section were Olivier Garraud, M.D. and Ph.D. (from French Guiana); and T. Moore, M.D. (a Clinical Associate from LCI). Coming to work with Dr. Sacks were G. Modi, a sandfly expert who had been at



NAMRU-3 and B. Butcher, Ph.D., from a post doc position at Stanford. A. Shakarian, a recent Ph.D. from the Medical College of Virginia, came to work with Dr. Dwyer, and G. Yap, a recent Ph.D. from the Univ. of Montreal (originally from the Philippines) came to work with Dr. Cheever. Departing Fellows included M. Alleman, Ph.D. who took an academic position at Bucknell University, I. Eltoum, M.D., who started an anatomic pathology residency at George Washington Univ. Hospital and N. Raghavan, Ph.D. who took a position at Johns Hopkins School of Hygiene. Y. Miyahira, M.D., took another post doctoral position at New York University with Dr. Nussenzweig and J. Actor, Ph.D. accepted a position with Cyt-Rx in Georgia. I. Oswald, Ph.D., returned to her home institution in France. L. Carrera-Ferrer, D.V.M. and Ph.D. took a position at Cenain Campus Politecnico in Ecuador, and M. Williams Ph.D., took a position with industry (Diacrin, Inc.) in Massachusetts.

The third annual Gorgas Memorial Leon Jacobs endowed lectureship sponsored by LPD was given by Jack Remington, M.D. of the Scripps Research Foundation and Stanford University in November. In discussing some of his research on toxoplasmosis he also reminisced about his research training with Dr. Jacobs at NIH.

In spite of limited funds for foreign travel, LPD research in foreign countries continued to be active last year. Dr. Neva made multiple trips to Honduras where he initiated and is monitoring a topical treatment trial of cutaneous leishmaniasis in collaboration with Dr. Carlos Ponce of the Ministry of Health. Dr. Neva and Albert Gam also were in Salvador, Bahia, Brazil to initiate a study with Dr. Edgar Carvalho on strongyloidiasis. Drs. Ottesen and Mahanty went to Madras, India to continue their filariasis studies and they were followed by Dr. Gopinath. Dr. McCarthy made a follow-up visit to the filariasis project site in the Cook Islands. Drs. Elson, Zimmerman and Nutman went to Ecuador for work on onchocerciasis.

### HONORS AND AWARDS

All of the permanent professional staff, and many of the senior nontenured staff serve on Editorial Boards of scientific journals and/or frequently review manuscripts for journals. Such activities reflect the high standing of LPD scientists by professional peers. Also many of the staff serve on Ad Hoc Committees for granting agencies or foundations, and are invited to participate in workshops and scientific meetings. Such activities are generally not cited here individually unless felt to deserve special mention.

Dr. Alan Sher was the recipient of the Superior Service Award and Dr. David Sacks received the NIH Director's Award during the past year. Dr. Nash was invited to give one of the major talks at the International meeting on *Giardiasis* that was held in Australia.





## Host-Parasite Relations Section

Genomic organization of *Giardia* parasites. Conditions have now been worked out to induce encystment of *Giardia* parasites in a reproducible manner. With hybridomas that have been produced to cyst wall proteins or to secretory vesicles, it will be possible to screen cDNA libraries from encysting trophozoites to identify proteins involved in vesicular transport and/or the process of encystment. DNA transcripts begin to appear about 90 minutes after organisms are exposed to encystment medium, and one cyst wall protein has already been characterized.

The genomic organization of several variable surface proteins (VSPs) representing different groups of *Giardia* parasites was found to differ. However, one specific area was responsible for most of the divergence between genes (Mowatt and Nash)

Biochemistry of *Giardia*. The presence of zinc in the variable surface proteins (VSPs) of giardia parasites was determined by micro X-Ray analysis at a concentration of 0.5 mM. The presence of some iron, as well as zinc in native VSP was also confirmed. No carbohydrates were detected in native VSP (Nash and Mowatt)

As reported last year, insulin-like growth factors 1 and 2 (IGF-1 and IGF-2), which are components of Cohn fraction IV-1 human blood proteins, were found to be short-term growth factors for *Giardia*. In follow-up studies of this relationship an IGF-1 like receptor was demonstrated in *Giardia*, suggesting some role for its presence. Some preparations of Cohn fraction IV-1 differ in their ability to replace serum in TYI-S-33 growth medium for *Giardia*. This is now considered to be due to presence of antibodies directed against the surface of the parasite because inhibitory effect could be eliminated following absorption of Cohn fraction IV-1 with Staph A or after absorption with fixed whole trophozoites. (Lujan, Mowatt and Nash)

*Giardia* appears to be a good example of a primitive eukaryote for study of Golgi apparatus function in secretion of VSP's and cyst wall proteins. Both proteins were inhibited by the chemical, brefeldrin, which affects secretory mechanisms of higher eukaryotes. However, VSP's are produced in trophozoites that have no identifiable Golgi apparatus. Cyst wall proteins are secreted in specific vesicles in a Golgi-like structure near one of the two nuclei of the organism. This suggests that the two nuclei of *Giardia* may not be functionally equivalent. (Lujan, Mowatt and Nash)



Modified casein-free medium found for growth of amebae. One of the components of standard axenic medium for growth of amebae developed by Diamond of LPD, Casein Digest Peptone (CDP), has been a chronic problem due to its inter-lot variability in supporting growth of the parasite. Furthermore, no new lots of CDP could be found that supported adequate growth of the parasite. In the process of testing other peptone products that might replace CDP it was found that CDP could be eliminated completely by increasing the concentration of Yeast Extract in the medium. This casein-free medium, named YI-S, supports the growth of *E. histolytica* to the same levels as the previous standard medium, TYI-S-33.

Several of the most widely used isolates of *E. histolytica*, as well as other species of protozoa, have been grown in medium YI-S through a number of subcultures and found to compare with the old medium TYI-S-33. Several fresh isolates of *E. histolytica* were adapted to axenic growth successfully with the new medium. YI-S medium also supported the growth of one isolate of *Trichomonas vaginalis*, and in a slightly modified form for several isolates of *Giardia*. (Clark and Diamond)

Further work on DNA analysis of *Entamoeba* species. With the study of additional isolates and species of *Entamoeba*, including *E. polecki* and *E. coli*, significant intra-specific genetic variation has been observed. This complicates using ribosomal DNA analysis (ribotyping) for identification of species. The greatest differences have been found between species that produce cysts with differing numbers of nuclei. (Clark)

What happened to the mitochondrion in amebae? Since no structural organelle resembling a mitochondrion has been identified in *E. histolytica*, it has been assumed that amebae lack this organelle. However, two genes encoding proteins that are associated with the mitochondrion in other eukaryotes (pyridine nucleotide transhydrogenase and chaperonin hsp60) have been demonstrated in *E. histolytica*. Sequence and genomic structure analysis of the 2 genes is underway. This *hsp60* gene has also been found in *E. dispar*, the non-pathogenic close relative of *E. histolytica*. (Clark)

Cytokine modulation of schistosome egg granulomas depends upon origin of the eggs. The administration of exogenous IL-12 dramatically decreases the size of pulmonary granulomas which develop around eggs injected intravenously. The same treatment with IL-12 of mice that develop granulomas around eggs as a consequence of infection with *S. mansoni* has little or no effect upon size of the granulomas. Administration of IFN- $\gamma$  inhibits or blocks the IL-12 effect on lung granulomas, but has no effect on liver granulomas. Anti-IL-2 and anti-L-4 antibody treatment of mice also markedly down regulate pulmonary granulomas, but do not affect hepatic granulomas in infected animals. Since eggs injected via the portal vein react like those in the lung model to cytokine treatment, it was concluded that the site of the granulomas is not crucial. Additional evidence for this conclusion is the fact that eggs can be shunted to the lungs by creating a portocaval shunt, and in this instance both



hepatic and pulmonary granulomas are resistant to anti IL-4 down regulation. The difference between eggs laid by worms and eggs recovered from infected tissue and then injected is believed to be due to a poorer antigenic quality of injected eggs (Cheever, Wynn, Oswald and Sher)

Quantitative parasitology of schistosome infection. By digestion of organs and tissues of mice infected with only a single pair of schistosomes, and counting numbers of eggs recovered, it is possible to estimate fecundity of different strains or species of parasite. A single worm pair of *S. mansoni* produced about 330 eggs/day/female. *S. japonicum* laid over 2000 eggs/day/female early in infection, but the rate decreased with time to less than 1000 per day after one year. The fecundity of worms in mice was not affected by treatment with recombinant cytokines TNF- $\alpha$  or IL-12, or by treatment with various anti-cytokine antibodies (to IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, IL-5 or IL-10). (Cheever).

### Biophysical Parasitology Section

Further observations of synchronization of DNA synthetic cycle of *T. cruzi*. Last year it was reported that a variety of *Kinetoplastidae* including *T. cruzi*, could be synchronized in the G<sub>1</sub> phase of the DNA synthetic cycle after exposure to relatively high concentrations of hydroxyurea (HU) of 5 to 20 mM. For synchronization, exposure of cells to HU for approximately one doubling time is required; exposure to two or more doubling times is lethal to the cells. Serum free cultures can also be synchronized with HU. This facilitates recovery of substances related to the cell cycle without interference of a high serum protein background. Some success, varying widely among different stocks of *T. cruzi*, has been achieved in adaptation to growth in serum free media. (Dvorak).

Use of microcalorimetry to study biophysical processes in protozoa. An attempt is being made to detect changes in heat produced by cells as they undergo metabolic and biological reactions. Using a custom-built microcalorimeter developed in the Biomedical Engineering and Instrumentation Program the heat output from metabolic activity of *Crithidia luciliae* was detected and measured. This may be a method of gaining insight into biological reactions unobtainable by other physical methods.

### Immunology and Cell Biology Section (Leishmania)

Developmental biology of *Leishmania*. For the last several years this project has focused upon the changes that occur in the surface lipophosphoglycan molecule on leishmanial promastigotes as the parasite develops to the infective stage in the gut of the sandfly. The mechanism by which *L. donovani* becomes infective in the gut of its vector, *P. argentipes*, is attributed to folding and clustering of extended phosphoglycan chains so they





no longer bind to the midgut. Comparison of a large number of vector/parasite pairs revealed that species specific differences in vectorial competence were in every case directly correlated with the ability of the promastigotes to attach to the sandfly midgut. Surprisingly, *P. argentipes*, the proven vector of *L. donovani* in India, was permissive to all of the other species. (Sacks, Pimenta, Modi, Rowton of WRAIR, and Turco of Univ. of Kentucky).

Does *L. tropica* also cause visceral leishmaniasis in India? Previous annual reports have noted that scattered cases of visceralizing leishmaniasis caused by *L. tropica* were discovered in military returnees from the Persian Gulf. Unexpected reactions to a monoclonal antibody were observed with a leishmanial isolate from a typical case of visceral leishmaniasis from northeast India. This prompted a review of other isolates from our previous studies in Patna, India. Four isolates were found to be *L. tropica* on the basis of typing with monoclonal antibodies and isoenzymes. If the presence of *L. tropica* can be confirmed as a cause of kala azar in India in further and more extensive investigation, it will provoke a re-evaluation of the epidemiology of kala azar in India (Sacks, Kenney and Neva of NIH, Kreutzer of Youngstown State Univ. and Sinha and Saran of Patna, India).

Trial of topical therapy of cutaneous leishmaniasis in Honduras. The atypical, non-ulcerating cutaneous leishmaniasis recently described from Honduras is treated with injections of Glucantime, an expensive pentavalent antimony compound. Since topical application of paromomycin (a poorly soluble aminoglycoside antibiotic) has been reported to be effective in treatment of cutaneous leishmaniasis due to *L. major*, a trial of topical paromomycin was initiated in Honduras. The trial is a double-blind, placebo controlled trial that will evaluate 3 daily applications of drug or placebo for 4 weeks. One group of 26 patients has entered the trial and another group of about 25 will soon be started (Neva and Ponce of Ministry of Health, Honduras).

Surface membrane enzymes of leishmanial parasites. The quest to "nail down" the 3' nucleotidase (3'NT) membrane enzyme of leishmanial parasites has finally been realized. This is the membrane enzyme found only in trypanosomatid organisms. The gene was cloned, fully sequenced, and shown to be present as a single copy in the parasite genome. The biologic significance of the enzyme and why it is present only in one class of protozoa remains to be evaluated (Debrabant and Dwyer). A full length gene for one of the secretory acid phosphatases (SACP), which was only partially identified earlier, has been cloned. (Shakarian and Dwyer).

Identification and molecular characterization of genes involved in leishmanial development and differentiation. Identification of genes involved in leishmanial development and transformation from one stage to another is greatly facilitated by several procedures already in use. One is the technique for in-vitro culture of amastigote forms, free of mammalian host cells, of several different species of parasite. The other procedure is the use of hydroxyurea to synchronize cell populations, which has been developed by Dvorak for





trypanosomes. Several genes highly expressed in *L. donovani* amastigotes have been identified and sequenced. Also, a series of genes which are transiently expressed during amastigote to promastigote transformation has been identified (Joshi, Nakhasi and Pogue of FDA and Dwyer).

### Immunology and Cell Biology Section (Schistosomes and Toxoplasma).

#### Additional aspects of the inhibitory effect of IL-12 on schistosome egg granulomas.

The circumstances under which granuloma formation around schistosome eggs was inhibited in mice are described earlier in Cheever's report. Changes in other cytokines are reported here. Administration of exogenous IL-12 was associated with increased mRNA levels in the lungs of INF- $\gamma$ , IL-2, IL-10 and IL-12, while mRNA expression of IL-4, IL-5, and IL-6 was suppressed. Mice sensitized with eggs in combination with IL-12 to precommit them toward a Th1 response developed minimal granulomas. This suggests that vaccination with antigen plus IL-12 may prevent pathology resulting from Th2 cytokine production (Wynn, Oswald, El Toun and Cheever).

#### Effects of upregulatory and downregulatory cytokines on vaccine-induced immunity.

Previous work suggests that murine immunity to schistosomiasis is dependent upon induction of Th1 cytokine responses that activate macrophages and endothelial cells to kill schistosomes in-vivo. Therefore, IL-12 was tested and found to enhance resistance to challenge infection when given as an adjuvant I.P. with irradiated cercariae. Several other previous vaccine studies in which protection was poor or not achieved were re-examined for the possible role of downregulatory cytokines. One of these studies involved P strain mice which were found to produce high levels of the downregulatory cytokines IL-4 and IL-10. These cytokines prevented macrophages from being activated to kill schistosomulae. The other vaccine study which used BCG plus killed schistosomulae by different routes of administration was found to stimulate the downregulatory cytokine, TGF- $\beta$  when the non-protective route was used (Wynn, Oswald, Williams and James).

Mechanism of killing schistosomulae by cytokine-activated human macrophages and endothelial cells. It was previously shown that endothelial cells as well as activated macrophages were capable of cytotoxic activity against *S. mansoni* schistosomulae. For murine cells the mechanism of toxicity is regulated by cytokines and involves the production of nitric oxide (NO). Recently it was shown that human endothelial cells and monocyte derived macrophages are also able to kill this target upon cytokine activation. However, the human effector cell system involves a novel nitric oxide and reactive oxygen independent pathway. The biochemical basis of the human effector system is under investigation. (Oswald, Wynn and James).



Mechanism of monokine induction by *T. gondii*. INF- $\gamma$  synthesis by spleen cells after exposure to parasite extracts is dependent upon production of two monokines, IL-12 and TNF- $\alpha$ , by parasite stimulated macrophages. Since these results can be the consequence of macrophage activation by contaminating lipopolysaccharide (LPS), it was important to rule such a mechanism out. Therefore, the activation of six LPS-inducible genes and tyrosine phosphorylation was examined. Although the toxoplasma extract induced expression of several of the genes and tyrosine phosphorylation in a manner similar to LPS, these functions were not affected by LPS inhibitors, indicating that the macrophage response to parasite extract could not be attributed to LPS contamination. In related work, preliminary fractionation studies of *T. gondii* antigens suggest that the parasite antigen ligands responsible for TNF- $\alpha$  and IL-12 induction may be distinct. The potential importance of this finding is that a specific parasite fraction could stimulate IL-12 production selectively, without a TNF- $\alpha$  response, thereby avoiding the pathologic consequences of the latter cytokine. (Vogel of Wistar Institute, Sher and Coligan of NIH).

Demonstration of a superantigen in *Toxoplasma*. In examining capacity of non-immune mouse spleen cells to respond in-vitro to several types of *Toxoplasma* antigens, high levels of proliferation as well as INF- $\gamma$  secretion were found to occur. Based upon several criteria, these responses appeared to be driven by a superantigen present in the parasite. For example, the 3-fold expansion of T cells stimulated by *T. gondii* showed expression of V $\beta$ 5 chain of the T cell receptor. Also, a survey of lymphocytes from in-bred mouse strains revealed an inverse correlation between antigen-induced proliferation and genetic deletion of V $\beta$ 5. Furthermore, transfected and paraformaldehyde fixed fibroblast lines were able to bind *T. gondii* antigen and induce T cell proliferation, demonstrating that the response can be mediated by allogeneic class II molecules, and that it does not require internalization by the cell and antigen processing. Interestingly, after one week of culture with antigen, up to 70 per cent of the expanded V $\beta$ 5-expressing cells were CD8 positive. Superantigen driven expansion of INF- $\gamma$ , secreting CD8+ lymphocytes may play a role in the mainly INF- $\gamma$  dependent cell-mediated immunity characteristic of infection with *Toxoplasma*. Moreover, the genetically determined response to this parasite antigen may explain variable manifestations of acquired and congenital toxoplasmosis in different individuals (Denkers and Sher).

Role of IL-12 in chronic toxoplasma infection. Peritoneal macrophages exposed either to live parasites or soluble tachyzoite antigens produced IL-12 protein. In mice, increased expression of mRNA for IL-12 in spleen and peritoneal cells was detected as early as 2 days post infection. Treatment of infected mice with anti-IL-12 monoclonal antibody increased susceptibility to acute infection. In contrast, neutralization of endogenously produced IL-12 had no effect when given during chronic infection. Thus, macrophage derived IL-12 appears to play a major protective role in acute *T. gondii* infection, but may not be required to maintain established immunity in the chronic stage (Gazzinelli).



## Clinical Parasitology Section

### Molecular techniques to identify specific and novel recombinant filarial antigens.

Over the last few years it has been possible to obtain well characterized serum specimens from selected patients with various filarial infections, plus microfilariae or adult worms, and occasionally infective larvae from patients or experimental infections. With these materials it has been possible to produce cDNA expression libraries and screen for recombinant products that react with individual or pooled sera. For example, with serum from individuals immune to onchocerca infection from an endemic area in Ecuador, 2-D immunoblots of infective stage *B. malayi* larvae were screened and 8 candidate antigens were identified (McCarthy and Nutman). Again, using 2-D gel electrophoresis two allergens with apparent molecular weights of 23 and 25 kD were recognized by immunoblot analysis. These antigens were identified as microfilarial proteins and were shown to induce IgE in-vitro and IL-4 in T cells of sensitized patients (Nutman). Additional recombinant products have been identified by screening with serum from patients with elevated IgE levels. Five of these recombinants are of particular interest because they are capable of inducing IgE and IgG4 in-vitro, and do so in an IL-4 dependent manner (Garraud and Nutman). Starting with 25 individual recombinants four were finally selected as being specific as diagnostic for individuals with lymphatic filariasis. These antigens are currently being purified for development of an Elisa test that could be used in the field. (Nutman).

Phenotypic characterization of cells producing cytokines. Using intracellular staining for cytokines in combination with three-color flow cytometry, the CD4+ CD27+ population of cells from both helminth infected patients and normal donors was found to have a 2 to 8-fold higher frequency of cells producing cytokines than did the CD4+ CD27- population, suggesting that the CD4+ CD27- subpopulation defines cells in a later stage of differentiation. Also, a low but finite proportion (0.2 to 8%) of CD4+ CD27- cells could produce both IL-4 and INF- $\gamma$ , and >60 percent of IL-5 producing cells also produced IL-4, but only 3 to 54 percent of IL-4 producing cells also produced IL-5. So human Th2 cells can produce IL-4 and/or IL-5. (Elson and Nutman of LPD, Prussin of LCI).

Use of the polymerase chain reaction (PCR) for diagnosis of filarial infection. Since antibody responses in lymphatic filariasis cannot distinguish between active infection vs. past or present exposure, the most reliable indicators of infection are (1) direct detection of microfilariae (mf) in the blood (or, rarely, adult parasites in lymphatics) or (2) detection of circulating parasite antigen. To explore detection of filarial DNA in the blood by PCR amplification, 60 patients with *Bancroftian filariasis* from Brazil, India and Polynesia were studied along with 50 non-endemic controls. The SapI repeated DNA sequence specific for *W. bancrofti* was PCR amplified with use of appropriate primers. No non-specific reactions were found in the normals, and virtually all individuals with mf in their blood were positive





by PCR. But whether the PCR reaction is more sensitive than antigen detection in mf negative cases is not yet clear (McCarthy, Nutman, Ottesen).

In the case of onchocerciasis specific diagnosis is generally based upon detection of mf in the skin of infected patients. A PCR-based method for detecting the 0150 repeated DNA sequence from *O. volvulus* was developed and then configured into a convenient Elisa format. Results from more than 300 patients and controls indicate that this technique is more sensitive (as well as completely specific) than all previous diagnostic methods. It will likely become the new "gold standard" for discriminating between non-infected and those with active *O. volvulus* infections. (Zimmerman, Nutman and Elson).

Therapy of lymphatic filariasis. The dose and regimen required for effective treatment with diethylcarbamazine (DEC) are not clearly defined because of difficulty in determining if adult worms have been eradicated. An assay for circulating antigen is available, but in studies to date, antigen levels have rarely fallen to zero after treatment. Therefore, as part of the Cook Island studies the microfilaricidal effect of DEC and potential of the circulating antigen assay were evaluated. 24 infected individuals were treated with a one week course of DEC (8mg/kg/day) either twice with a 15 month interval, or monthly for 6 months, followed by a course at 15 months. Follow-up at 18 months indicated both regimens gave equivalent results with circulating antigen assays negative in 20/24 patients. (McCarthy and Ottesen).

Studies in India and Brazil on the use of ivermectin filariasis indicate that higher doses of ivermectin (400µg/kg) are more effective in sustaining long-term reductions in microfilaremia than lower doses used earlier. Also, it was found that single dose DEC treatment can be essentially as effective as single dose ivermectin treatment when patients are followed for up to two years post-treatment. However, a combination of single-dose ivermectin and single-dose DEC probably gives the most effective results. Trials continue to optimize regimens for large-scale control programs. (Ottesen)





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00094-35 LPD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

*Entamoeba histolytica*: Cellular Physiology and Molecular Diagnostics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L.S. Diamond Section Head (retired 01/03/94) LPD, NIAID  
 T.E. Nash Section Head LPD, NIAID

Others: C.G. Clark Staff Fellow LPD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Parasite Growth and Differentiation

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The ability to mass cultivate *Entamoeba histolytica* under axenic conditions is crucial for many types of study on this organism to be undertaken. A major component of the current axenic medium, TYI-S-33, is Casein Digest Peptone. Recently a critical situation arose as no new lots of CDP could be found that supported adequate amebal growth. We have now developed a casein-free medium, YI-S, that supports growth of *Entamoeba histolytica* and other *Entamoeba* to the same levels as TYI-S-33.

TYI-S-33 was also widely used for cultivation of *Trichomonas vaginalis* and *Giardia*. YI-S was tested for its ability to sustain the growth of one isolate of *Trichomonas* and two of *Giardia*, again with good results. The availability of a casein-free medium will remove the single greatest source of variability in the axenic culture of *E. histolytica*.

We have continued to examine the taxonomy of the genus *Entamoeba* at the molecular level. A number of additional isolates and species, including *E. polecki* an organism with zoonotic potential, have been studied. Significant intraspecific genetic variation has been observed in certain species.

In contrast to current dogma, we have found that *Entamoeba histolytica* appears to have organelles related to the mitochondrion. Sequence and genomic structure analyses of two *Entamoeba* genes encoding proteins thought to be associated with this organelle are underway.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00099-24 LPD

PERIOD COVERED

September 30, 1993 to October 1, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biophysical Parasitology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Others:	J. A. Dvorak	Res. Microbiologist	LPD, NIAID
	J. P. McDaniel	Biologist	LPD, NIAID
	D. B. Rainey	Biological Lab. Tech.	LPD, NIAID
	Y. Mihahira	Fogarty Fellow	LPD, NIAID
	C. P. Mudd	Senior Engineer	ACE, BEIP
	N. Galanti	Head, Mol. Biol.	Univ. Chile, Santiago,
	D. P. Remeta	Senior Staff Fellow	Lab. Biochem., NHLBI
	S. E. Abdallah	Guest Worker	LPD, NIAID

COOPERATING UNITS (If any)

Biomedical Engineering and Instrumentation Program, DRR  
Laboratory of Biochemistry, NHLBI  
Dept. of Cell Biol. & Genetics, University of Chile

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Physiology and Biochemistry

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.5

PROFESSIONAL:

2.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project is concerned with analyses of the genetic diversity of medically important parasitic protozoa and its implications to the epidemiology, course, and diagnosis of disease. The project has become increasingly involved in elucidation of diversity at the DNA level. We are refining a method we developed to synchronize the DNA synthetic cycle of trypanosomatids using very high levels of hydroxyurea. We will utilize the technique for the production of large quantities of synchronized cells for elucidation and analyses of cell cycle-specific and developmental stage-specific substances. We have identified an oligonucleotide which appears to differentiate between stocks of *Trypanosoma cruzi* at the DNA level. We are attempting to complete feasibility studies of the use of a microcalorimeter to study metabolic processes in intact protozoa.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00102-20 LPD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pathogenesis of Disease Caused by Infection with Intracellular Parasites

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	F.A. Neva	Chief	LPD, NIAID
Others:	D. Sacks	Research Microbiologist	LPD, NIAID
	A. Gam	Biol. Lab. Tech. (Micro.)	LPD, NIAID
	R. Kenney	Sr. Staff Fellow	LPD, NIAID
	G. Lanzaro	Guest Researcher	LMR, NIAID

COOPERATING UNITS (If any)

Dept. Biology, Youngstown State Univ. (R. Kreutzer); Walter Reed Army Med. Center (C. Oster and A. Magill); National Naval Med. Center (C. Ohl); Ministry of Health, Honduras (C. Ponce); Leishmaniasis Research Program, WHO/TDR, Geneva (F. Modabber).

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Cell Biology and Immunology

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.5

PROFESSIONAL:

1.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project focuses upon the different clinical forms of leishmanial infections in humans, immune responses to the parasite, with particular reference to cell-mediated responses and the cytokine regulation of these responses, and characteristics of the causative parasites.

Ten patients were referred to NIH for suspected leishmaniasis. Six were U.S. Peace Corps volunteers or tourists, and four were military personnel who had been in the Persian Gulf region and were suspected of having visceralizing leishmaniasis. All patients treated with Pentostam (pentavalent antimony) experienced asymptomatic rises in serum amylase and lipase.

A trial of topical treatment with paromomycin for atypical, nonulcerating cutaneous leishmaniasis was initiated in Honduras. This is a randomly selected, double-blind, placebo-controlled study comparing 15% paromomycin plus 10% urea in soft paraffin vs. paraffin alone as placebo. Twenty six cases were enrolled in the study in July, 1994.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AI 00161-17 LPD

## PERIOD COVERED

October 1, 1993 to September 30, 1994

## TITLE OF PROJECT

Immunochemistry of Parasitic Diseases

## PRINCIPAL INVESTIGATOR

T.E. Nash

Medical Officer

LPD, NIAID

Others: M.M. Mowatt, Staff Fellow, LPD, NIAID; D.H. Lujan, Visiting Fellow, LPD, NIAID; L. Byrd, IRTA, LPD, NIAID; A. Sher, LPD, NIAID; M. Chance, Asst. Professor, Albert Einstein College of Med.; R. Leapman, Biomedical Engineer, NCCR; H. Stipps, Research Assoc. Prof., Tulane Univ.; L. Helman, Pediatrics Branch, NCI; M. Heyward, Assoc. Prof., Univ. of California, S.F.; B. Gottstein, Institute of Parasitology, University of Zurich, Switzerland; S. Erlandsen, University of Minnesota

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Parasitic Diseases

## SECTION

Host-Parasite Relations Section

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.5

## PROFESSIONAL:

2.0

## OTHER:

1.5

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

VSPs are the major surface protein of *Giardia lamblia* and undergo antigenic variation. One VSP was purified and analyzed. The native protein contained large quantities of Zn and a lesser amount of Fe. The amino terminal sequence was cleaved off as expected and carboxyl terminal region was present indicating it likely serves as the membrane-spanning region. No carbohydrates were detected. Insulin-like growth factor is a growth factor for *Giardia* and stimulates growth by way of an IGF-1-like receptor on the surface of trophozoites. The receptor was mammalian-like, reacted with antibodies made to human receptors, and had intrinsic tyrosine kinase activity. This is the first demonstration of this type of receptor in an organism lower than fish and suggests unique host-parasite interactions. Lipids are essential growth factors for *Giardia* in serum. Some preparations also contain inhibitory substances which are likely antibodies.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00162-18 LPD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biochemical Cytology of Host-Parasite Interactions in Parasitic Protozoa

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

	D.M. Dwyer	Supervisory Microbiologist	LPD, NIAID
Others:	A. Debrabant	Visiting Fellow, FIC	LPD, NIAID
	J.A. Dvorak	Supervisory Microbiologist	LPD, NIAID
	S.L. Ellis	Biologist	LPD, NIAID
	P.F.P. Pimenta	Guest Investigator	LPD, NIAID
	G.J. Russell	Visiting Fellow, FIC	LPD, NIAID
	D.L. Sacks	Supervisory Microbiologist	LPD, NIAID
	A. M. Shakarian	IRTA Fellow	LPD, NIAID

COOPERATING UNITS (if any)

CBER, DHP, FDA, (M. Joshi, G. Pogue & H. Nakhasi); DMID, NIAID (M. Gottlieb); Dept. Biochem., Univ. Kentucky (S. Turco); London School Tropical Med. & Hyg. (R. Harris & P. Kave).

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Immunology and Cell Biology

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

5.5

PROFESSIONAL:

4.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The cell and molecular biology of both *Leishmania* and *Toxoplasma* are investigated as models of intracellular parasitism. Emphasis is placed on characterizing both the biochemical functions and gene structure of their surface membrane and secreted proteins toward defining the roles of these constituents in parasite survival and development.

A gene encoding the unique, trypanosomatid surface membrane enzyme, 3'-nucleotidase has been identified, cloned and fully sequenced from *L. donovani*. Similarly, full length genomic clones from an *L. donovani* secretory acid phosphatase gene family are being sequenced. The role of this enzyme within infected macrophages is also being investigated. Methods were devised for the continuous *in vitro* cultivation of large quantities of infectious amastigotes (Am-) from *L. donovani* and several other species. These Am- are being characterized with regard to their cell biology and biochemistry. At the molecular level, several genes have been identified which are uniquely or differentially expressed by Am- and these are being used as probes to study parasite gene-regulated differentiation and cell cycle development. Further, a portion of a gene for both a surface membrane 5'-nucleotidase and a "secretory" chitinase have been identified and sequenced from PCR. Finally, a unique 5'-nucleotidase was identified in the surface membrane of *Toxoplasma gondii* tachyzoites and a PCR-generated probe is being used to identify the gene for this protein.

The current results are of relevance toward the development of new diagnostic, chemotherapeutic and immunoprophylactic agents against these important human pathogens.



October 1, 1993 to September 30, 1994

## Immunoregulation and Immune Recognition in Filariasis and Non-Filarial Disease

PI:	T.B. Nutman	Senior Investigator	LPD, NIAID
Others:	E.A. Ottesen	Senior Investigator	LPD, NIAID
	S. Mahanty	Senior Staff Fellow	LPD, NIAID
	L. Elson	Visiting Fellow, FIC	LPD, NIAID
	P. Zimmerman	NRC Fellow	LPD, NIAID
	S. Mawhorter	Clinical Associate	LPD, NIAD
	S. Shaw	Senior Investigator	EIB, NCI
	C. Prussin	Clinical Associate	LCI, NIAID

## COOPERATING UNITS (if any)

Dept. of Clinical Investigation, Hospital Vozandes, Quito, Ecuador (Dr. R. Guderian); Univ. National de Benin (Dr. Massouboudgi); Anna Univ., Madras, India (Dr.K. Jayaraman); Immunology Unit, University of Chicago (G. van Seventer)

## LAB/BRANCH

Laboratory of Parasitic Diseases

## SECTION

Clinical Parasitology Section

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20892

## TOTAL STAFF YEARS:

3.5

## PROFESSIONAL:

3.3

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this project is to delineate the mechanisms involved in regulating immune responses in filarial and nonfilarial disease states. Immunoregulatory studies have examined the phenomenon of antigen-specific anergy in microfilaremic patients by showing this anergy to be a result of the production of the antiproliferative cytokine, IL-10. Filter immunoplaque assays for the major cytokines have been developed and used to demonstrate that in helminth infections of humans there is an expansion of Th2 CD4+ cells. The phenotypic characterization of these Th2 CD4+ cells has shown them to be CD45RO+ HLA-DR+ CD27-; a novel method for intracellular staining for cytokines has been used to assess further the frequency of these cells. Similarly, new ways of assessing eosinophil activation have also been developed.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AI 00246-12 LPD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Studies on the Genome and Surface of *Schistosoma mansoni*

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Alan Sher

Section Head

LPD, NIAID

COOPERATING UNITS (if any)

Molecular Vaccines, Inc., Gaithersburg, Maryland

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Immunology and Cell Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Terminate



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AI 00251-13 LPD

## PERIOD COVERED

October 1, 1993 to September 30, 1994

## TITLE OF PROJECT

Immunologic Studies on Schistosomiasis

## PRINCIPAL INVESTIGATOR

A. Sher	Section Head	LPD, NIAID
T. Wynn	IRTA Fellow	LPD, NIAID
I. Oswald	Special Volunteer	LPD, NIAID
M. Williams	Special Volunteer	LPD, NIAID
D. Jankovic	IRTA Fellow	LPD, NIAID
A. W. Cheever	Assistant Chief	LPD, NIAID
S. L. James	Program Officer	MIDP, IRP, NIAID
I. Eltoum	Visiting Fellow	LPD, NIAID
S. Fouad	Special Volunteer	LPD, NIAID

## COOPERATING UNITS (If any)

## LAB/BRANCH

Laboratory of Parasitic Diseases

## SECTION

Immunology and Cell Biology Section

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

4.0

## PROFESSIONAL:

3.0

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

The aim of this research program is to investigate mechanisms of protective immunity and immunopathology in schistosomiasis with the ultimate goal of immunologic intervention. Progress was achieved in the following areas during the year.

A) Prevention of egg granuloma formation with IL-12. The cytokine IL-12 was shown to block pulmonary egg pathology when given at the same time as granuloma formation and to prophylactically immunize against pathology when used in conjunction with egg immunization.

B) Effects of upregulatory and downregulatory cytokines on vaccine-induced immunity. IL-12 when given at the time of vaccination with irradiated cercariae was shown to markedly enhance protective immunity. Conversely, the reduced immunity of vaccinated P-strain mice and C57BL/6 mice immunized with dead antigen via the intramuscular or intravenous as opposed to protective intradermal routes was shown to be associated with the production of the downregulatory cytokines IL-4 and IL-10 and TGF- $\beta$ , respectively.

C) Mechanism of killing of schistosomula by cytokine-activated human macrophages and endothelial cells. Cytokine activated human macrophages were shown to kill schistosomula via a novel nitrogen oxide, respiratory burst independent mechanism.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00253-13 LPD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Immunological Responses to Filarial Infections

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E.A. Ottesen	Section Head	LPD, NIAID
Others:	T.B. Nutman	Senior Investigator	LPD, NIAID
	C. Steel	Biologist	LPD, NIAID
	S. Mahanty	Medical Staff Fellow	LPD, NIAID
	J. McCarthy	Visiting Fellow	LPD, NIAID
	P. Zimmerman	NRC Fellow	LPD, NIAID
	R. Gopinath	Visiting Worker	LPD, NIAID

COOPERATING UNITS (if any)

Indian Council of Medical Research, Madras, India (S. Tripathy, V. Kumaraswami); Anna University, Madras, India (K. Jayaraman); CPqAM/FIOCRUZ, Recife, Brazil (G. Drever, A. Coutinho); Health Department, Cook Islands (A. Guinea); (Cont below)

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Clinical Parasitology

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda Maryland 20892

TOTAL STAFF YEARS:

2.2

PROFESSIONAL:

2.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☒ (a1) Minors  
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

(Cooperating Units Cont.)

James Cook University, Queensland, Australia (P. Turner); Washington University, St. Louis, MO (G. Weil); Smith College, Northhampton, MA (S. A. Williams)

The goals of this project are to define the immunologic mechanisms responsible for resistance to filarial infection, acquisition of filarial disease, and the pathological reactions that develop in patients being treated for these infections.

From observations from an island population 17 years apart, individuals were seen to remain entirely free from infection with bancroftian filariasis, their landing greater certainty to classifying them as "putatively immune." Their serologic, lymphocyte proliferation, and PBMC cytokine responses to adult worms and microfilarial antigen were significantly greater than those of infected individuals. Analysis of possible protective immune responses to infective larval antigens is underway.

The development of overt lymphatic pathology in patients followed for 17 years did not correlate with either clearance of microfilaremia or enhanced immune responsiveness to parasite antigens. Predisposition to infection might relate to prenatal tolerization to microfilarial antigen identified in children 17 years after their mothers were diagnosed as being either microfilaremic or non-infected during pregnancy. Mechanisms of this immunological tolerance and genetic factors relating to immune and pathological responses are being explored.

The acute post-treatment inflammatory responses to DEC treatment of bancroftian filariasis and onchocerciasis have been shown to be related to serum levels of IL-6 and TNF. Similar detailed studies have been carried out on onchocerciasis patients being treated with ivermectin, and evaluation of the specimens is underway.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00256-13 LPD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Developmental Biology of *Leishmania* Promastigotes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. Sacks	Senior Investigator	LPD, NIAID
Other:	P. Pimenta	Visiting Associate	LPD, NIAID
	R. Kenney	Senior Research Investigator	LPD, NIAID
	E. Saraiva	Guest Researcher	LPD, NIAID
	G. Modi	Visiting Fellow	LPD, NIAID
	B. Butcher	Visiting Fellow	LPD, NIAID

COOPERATING UNITS (if any)

Dr. Sam Turco, Dept. of Biochemistry, University of Kentucky; Dr. Stephen Beverley, Harvard Medical School, Boston; Dr. Ed Rowton, Walter Reed Army Institute of Research, Washington

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Cell Biology and Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:

PROFESSIONAL:

5.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The life-cycle of *Leishmania* parasites within the sand fly vector includes the development of extracellular promastigotes from a noninfective, procyclic stage into an infective, metacyclic stage. These adaptations were explored in the context of the structure and function of the abundant surface lipophosphoglycan (LPG) on *Leishmania donovani* promastigotes. During metacyclogenesis, two important developmental modifications were observed. First, the size of the molecule is substantially increased due to a 2-fold increase in the number of phosphorylated disaccharide repeat units expressed. Second, there is a concomitant decrease in the presentation of terminally exposed sugars. The capping sugars on procyclic LPG were found to mediate procyclic attachment to the sand fly midgut, whereas these same sugars on metacyclic LPG failed to mediate metacyclic binding. And whereas intact metacyclic LPG did not inhibit procyclic attachment, depolymerized LPG inhibited as well as procyclic LPG, demonstrating that the ligands are normally buried. The exposure and subsequent masking of the terminal capping sugars explains the stage-specificity of promastigote attachment to and release from the vector midgut, which are key events in the development of transmissible infections in the fly.

Phlebotomine vectors are in some instances able to transmit only certain species of *Leishmania*. Comparison of a large number of vector/parasite pairs revealed that species-specific differences in vectorial competence were in every case directly correlated with the ability of promastigotes to attach to the sand fly midgut, the variable outcomes of which were controlled by structural polymorphisms in the surface lipophosphoglycan (LPG) of the parasite. The data suggest that at least some phlebotomine vectors differ with respect to the parasite recognition sites which they express, and that midgut adhesion is a sufficiently critical component of vectorial competence as to provide the evolutionary drive for LPG structural polymorphisms.

Mutants defective in expression of specific sugars involved in midgut attachment have been selected using lectins or antibodies which bind to the functional domains on LPG. LPG mutants have been obtained which fail to bind to the midgut or survive in the fly, and we are attempting to recover the defective genes by functional complementation.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00257-13 LPD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunology of Strongyloidiasis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	F. A. Neva	Chief	LPD, NIAID
Others:	A. Gam	Technician	LPD, NIAID
	T. Nutman	Senior Investigator	LPD, NIAID
	T. Nash	Senior Investigator	LPD, NIAID
	D. Alling	Special Asst. for Biometry	DIR, NIAID

COOPERATING UNITS (if any)

Bioqual, Inc. (T. Moskal); Northwestern University, Evanston, Illinois (P. Gann); Univ. of the West Indies, Jamaica (R. Robinson); Johns Hopkins University, Baltimore (N. Raghavan); Federal University of Bahia, Brazil (See below)

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Immunology and Cell Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.4

PROFESSIONAL:

0.9

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects    ☒ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Cooperating Units (Cont.)

(E. Carvalho, A. and M. Barral); Walter Reed Army Inst. of Research, Infect. Dis. and Prev. Med. (J. Stoute and B. Petrocelli)

This project deals with both the laboratory and clinical aspects of infection caused by the intestinal nematode, *Strongyloides stercoralis*. The laboratory research involves analysis and characterization of parasite antigens. The clinical studies focus upon the immune response of infected individuals, and factors that influence the immune response. Experimental infections with the parasite in a newly recognized animal host, the jird (*Meriones unguiculatus*), have been initiated recently.

The recombinant gene product from a cDNA library from 3rd stage parasite larvae was transferred to a pMAL vector. The recombinant product from the new vector is reactive by Western blot with sera from *S. stercoralis*-infected patients. It shows a high degree of sequence homology with collagen genes of *C. elegans*.

Collaborative clinical studies on patients infected with *S. stercoralis* were initiated in Bahia, Brazil. The immune response in patients also infected with the retrovirus, HTLV-1, is of particular interest. Serum samples as late as 1 1/2 years after Ivermectin or thiabendazole treatment of strongyloidiasis in Cambodian refugees were found to show a mixed pattern of response.

Several human strains of *S. stercoralis* were found to have a very low efficiency of infection for the jird (*Meriones unguiculatus*) in comparison to a parasite strain from the dog. Infection with the dog strain was enhanced by administration of prednisolone.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 AI 00347-12 LPD

PERIOD COVERED

October 1, 1993 - September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on Schistosomal Hepatic Fibrosis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

A.W. Cheever, Head, Host-Parasite Relations, LPD, NIAID

Others: R. Poindexter Bio. Lab. Tech., LPD, NIAID  
T. Wynn Immunology Sect., LPD, NIAID  
A. Sher Head, Immunology Sect., LPD, NIAID

COOPERATING UNITS (if any)

Department of Medicine, USUHS (Fred Finkelman) and USDA (Joseph Urban). Centro de Pesquisas Gonçalo Moniz, Salvador, Bahia, Brazil (Zilton Andrade).

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Host-Parasite Relations Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.2

PROFESSIONAL:

0.6

OTHER:

0.6

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Morbidity in schistosome-infected individuals is caused mainly by the immune response of the host to schistosome eggs deposited in the tissues. In chronic schistosomiasis, fibrotic sequelae to the inflammatory reaction to the eggs are responsible for most clinical disease.

Hepatic fibrosis and the granulomatous response to eggs of schistosome species pathogenic for man are studied in mice in relation to parasitologic parameters of infection. Cytokines play an important role in the genesis and regulation of the size of circumoval granulomas and in the fibrosis associated with them.

Pulmonary granulomas around intravenously injected *S. mansoni* eggs are frequently used to study the immunopathology of schistosomiasis. This system allows better manipulation of the mouse than one obtains with infected animals. In the past year we have found major differences in immunomodulation of granulomas in this "lung model" compared to infected mice. Anti-IL-2 and anti-IL-4 antibodies and administration of exogenous IL-12 have major effects on granuloma size in the lung model but little effect on hepatic granulomas in infected mice. Granulomas around eggs injected into the liver via the portal vein behaved like those in the lung model, suggesting that the site of the granuloma was not crucial. This was confirmed by creating portacaval vascular shunts in infected mice, shunting eggs in infected animals to the lungs. Anti-IL-4 antibodies did not affect these granulomas. Granulomas injected into the lungs of infected mice were downregulated by anti-IL-4, indicating that the vigorous response of infected animals was not responsible for the failure of anti-IL-4 to modulate responses in infected mice. We believe the crucial factor to be the quality of the eggs injected in the lung model. Defining the difference between granulomas in infected mice and those in the lung model is crucial to understanding the *in vivo* regulation of the granulomas.





**DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT**

**PROJECT NUMBER**

Z01 AI 00350-12 LPD

**PERIOD COVERED**

October 1, 1993 to September 30, 1994

**TITLE OF PROJECT**

DNA Analysis of Parasites

**PRINCIPAL INVESTIGATOR**

P.I.	T. E. Nash	Medical Officer	LPD, NIAID
Others:	M. M. Mowatt	Staff Fellow	LPD, NIAID
	J. Yee	Visiting Fellow	LPD, NIAID

**COOPERATING UNITS (If any)**

**LAB/BRANCH**

Laboratory of Parasitic Diseases

**SECTION**

Host-Parasite Relations

**INSTITUTE AND LOCATION**

NIAID, NIH, Bethesda, MD 20892

**TOTAL MAN-YEARS:**

3.5

**PROFESSIONAL:**

2.5

**OTHER:**

1.0

**CHECK APPROPRIATE BOX(ES)**

- ☐ (a) Human subjects ☒ (b) Human tissues (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

**SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)**

A cyst-protein gene was identified and sequenced. The predicted protein is a 26 kDa leucine-rich protein with 5 degenerate repeats. Monoclonal antibodies were produced to other cyst and secretory proteins. The genomic organization of the H7 VSP of isolate GS/M was determined. In contrast to the tail-to-tail arrangement of two identical 1267 VSP genes, there was only one VSP H7 gene. Another closely related H7 gene was identified and sequenced. One specific area was responsible for most of the divergence between the genes. The upstream untranslated regions were practically identical. The control of VSP transcription must take account of the identical upstream regions which usually contain controlling transcriptional elements.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00439-10 LPD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Clinical and Therapeutic Studies of Human Filariasis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E.A. Ottesen	Section Head	LPD, NIAID
Others:	T.B. Nutman	Senior Investigator	LPD, NIAID
	C. Steel	Biologist	LPD, NIAID
	S. Mahanty	Medical Staff Fellow	LPD, NIAID
	J. McCarthy	Visiting Associate	LPD, NIAID
	P. Zimmerman	NRC Fellow	LPD, NIAID
	L. Elson	Visiting Fellow	LPD, NIAID
	R. Gopinath	Visiting Researcher	LPD, NIAID

COOPERATING UNITS (if any)

Indian Council of Medical Research, Madras, India (S.P. Tripathy, V. Kumaraswami, R. Prabhakar); MGR Medical College, Madras, India (V. Vijayasekaran); Peace Corps Medical Office, Washington; Centro de Pesquisas Aggeu Magalhaes, (Cont.)

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Clinical Parasitology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.6

PROFESSIONAL:

1.5

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Recent clinical studies have defined the presence of individuals with natural immunity to infection lasting at least 17 years. Some infected individuals may remain microfilaremic over this same period of time without developing overt lymphatic pathology, but others develop such pathology that can develop either in the setting of persistent microfilaremia or after microfilaremia has cleared. Genetic factors appear to predispose one to develop tropical pulmonary eosinophilia syndrome; familial studies are underway to define the mechanisms.

PCR-based amplifications of parasite DNA in skin snips from patients with onchocerciasis has increased diagnostic sensitivity and specificity, and the assays have been configured into an ELISA format that is field applicable. Similar efforts are underway to detect parasite DNA in lymphatic filariasis, but sensitivity is not yet superior to diagnosis by parasitologic or antigen-detection means.

Because the Cook Islands population studied was relatively lightly infected, the treatment trial comparing 2 or 7 one-week courses of DEC given over 15 months showed equivalent therapeutic effects but, most importantly, showed for the first time that circulating filarial antigen is an excellent and readable marker of active bancroftian filarial infection. Other studies showed that single yearly doses of ivermectin or DEC are extremely effective in reducing microfilaremia in bancroftian filariasis, and either drug alone (or together) would be appropriate for control programs. In contrast, even 3 three-week courses of DEC could cure only about 2/3 of expatriates with loiasis; more effective chemotherapy is needed.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00487-08 LPD

PERIOD COVERED

October 1, 1993 - September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Quantitative Parasitology of Schistosome Infections

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

A.W. Cheever, Head, Host-Parasite Relations Section, LPD, NIAID

Others: R. Poindexter Bio. Lab. Tech., LPD, NIAID  
F. Lewis Biomedical Research Institute  
J. Mosimann ORI  
Z. Andrade Fogarty Scholar

COOPERATING UNITS (if any)

Centro de Pesquisas Gonçalo Moniz, Salvador, Bahia, Brazil (Zilton Andrade).  
Biomedical Research Institute, Rockville, MD (Fred Lewis).

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Host Parasite Relations Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.8

PROFESSIONAL:

0.4

OTHER:

0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Morbidity in schistosomiasis is caused by the host response to schistosome eggs which are deposited in the venous system and carried to the tissues. Pathology is proportional to the number of eggs laid and it is thus important to understand factors underlying worm fecundity and the extent to which fecundity is reflected by eggs passed in the feces, the measurable indicator of infection intensity in humans.

The rate of destruction of *S. mansoni* or *S. japonicum* eggs in the tissues of mice has proved to be so slow as to make it a negligible factor in calculating the fecundity of schistosomes. The fecundity (essentially = to eggs/day passed in the feces + eggs/day accumulated in the tissues) of *S. mansoni* in mice infected with a single pair of worms averages about 330 eggs/day/female and eggs passed in the feces reflect the intensity of egg laying in individual mice over a 1-year period of infection. *S. japonicum* lays over 2000 eggs/day/female early in infection but the rate of egg laying decreased with increased duration of infection to less than 1000 eggs/day/female 1 year after infection. The number of eggs in the feces decreases in parallel to the decrease in total egg laying. The fecundity of *S. mansoni* but not *S. japonicum* is decreased in SCID mice. *S. mansoni* frequently fails to mature in nude mice but worms which mature produce normal numbers of eggs.

We have not been able to affect the fecundity of worms in mice by treatment with recombinant cytokines (TNF- $\alpha$  or IL-12) or by treatment with anti-cytokine antibodies (to IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, IL-5, IL-6, IL-10).



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00494-08 LPD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analysis of T Cell Responses in Human Leishmaniasis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. Sacks	Senior Investigator	LPD, NIAID
Others:	R. Kenney	Senior Research Investigator	LPD, NIAID
	L. Carrera	Guest Researcher	LPD, NIAID
	F. Neva	Chief	LPD, NIAID

COOPERATING UNITS (if any)

Shyam Sundar, Benares Hindu University

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Cell Biology and Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

PROFESSIONAL:

OTHER:

3.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The critical immunological feature of visceral leishmaniasis is the complete absence of cell-mediated immunity to leishmanial antigens. Patients have been shown to have negative intradermal skin tests to Leishmanin, absent lymphocyte blastogenesis, and decreased IL-2 and IFN- $\gamma$  production in response to parasite antigens. Experimental models of visceral leishmaniasis suggest that the disease is characterized not by the lack of cytokine production per se, but by the production of potentially counterprotective cytokines. We sought to maximize assay sensitivity by using semiquantitative RT-PCR techniques to analyze cytokine mRNA extracted from lesional tissue (bone marrow and spleen). In preliminary data we provided evidence that IL-10 mRNA is present at relatively high levels in active kala-azar, being at or below the limits of detectability after treatment, and absent in uninfected controls. The cellular source of this potentially downregulatory cytokine is being investigated in patients from India by repeating the above experiments using bone marrow cells positively and negatively selected for B cells, T cells, or monocytes. In addition, we now have available for analysis splenic tissue from Indian kala-azar patients during the course of their therapy with antimony alone, or with antimony plus IFN- $\gamma$ .

The ability of *Leishmania* to evade the microbicidal activities of their host macrophages is obviously key to their successful parasitism. The manner by which they accomplish this is being investigated *in vitro* by studying the effect of infection on the production of proinflammatory cytokines involved in macrophage activation and intracellular killing. The parasites alone failed to trigger expression of any cytokine message. More importantly, costimulation of the macrophages with parasites and potent activators, LPS or killed mycobacteria, led to a striking downregulation of IL-1, TNF- $\alpha$ , NO synthase, and particularly IL-12. In contrast, IL-10, which is known to downregulate disease controlling TH1 responses, was either not affected or in some cases upregulated by the parasite. These results are consistent with the ability of the parasite to establish itself intracellularly during the early stages of infection and to persist by evading host immune responses.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER  
 Z01 AI 00512-07 LPD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Definition of Filarial Antigens

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	T.B. Nutman	Senior Investigator	LPD, NIAID
Others:	J.S. McCarthy	Visiting Associate	LPD, NIAID
	L.H. Elson	Visiting Fellow, FIC	LPD, NIAID
	O. Garraud	Visiting Fellow, FIC	LPD, NIAID
	P.A. Zimmerman	NRC Fellow	LPD, NIAID
	T. Moore	Clinical Associate	LPD, NIAID

COOPERATING UNITS (if any)

New England Biolabs, Beverly, Mass (F. Perler, L. McReynolds, Swiss Tropical Institute (E. Lobos), Johns Hopkins University, Baltimore, MD (Alan Scott, Nithya Raghavan); Imperial College of Science and Technology, London. (See below)

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Clinical Parasitology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

3.1

PROFESSIONAL:

2.4

OTHER:

0.7

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

(Cooperating Units Cont.)

(J. Bradley); New York Blood Center (S. Lustigman); Hospital Vozandes, Quito, Ecuador (Ronald Guderian)

The objectives of this project are to define and generate filarial proteins that are important in inducing parasite-specific immune responses in the human host and to understand, at a molecular level, the differences among related filarial species. Recombinant antigens and probes have been identified that a) encode immunoreactive and potentially protective molecules of *W. bancrofti*; b) can distinguish among related filarial species; c) identify repeated segments of the *W. bancrofti*, *O. volvulus* and *Loa loa* genome; d) are of potential diagnostic importance; and e) are responsible for the induction of immediate hypersensitivity type responses in filarial infections.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AI 00579-05 LPD

## PERIOD COVERED

October 1, 1993 to September 30, 1994

## TITLE OF PROJECT

Studies on Immune Regulation in Toxoplasmosis and Other Opportunistic Infections

## PRINCIPAL INVESTIGATOR

A. Sher

Senior Investigator

LPD, NIAID

## Others:

R. Gazzinelli

Senior Staff Fellow

LPD, NIAID

E. Denkers

IRTA Fellow

LPD, NIAID

L. Aslund

Special Volunteer

LPD, NIAID

J. Coligan

Chief

LMS, NIAID

M. Martin

Chief

LMM, NIAID

## COOPERATING UNITS (if any)

Uniformed Services University, Bethesda MD (S. Vogel);  
Wistar Institute, Philadelphia PA (G. Trichieri, M. Wysocka).

## LAB/BRANCH

Laboratory of Parasitic Diseases

## SECTION

Immunology and Cell Biology Section

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.5

## PROFESSIONAL:

3.0

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

The overall aim of this project is to analyze the immune response to *Toxoplasma gondii* and other opportunistic infections in order to define which cellular immune components and parasite target antigens are involved in the control of infection and its breakdown in immunocompromised hosts.

Progress was made this year in the following areas:

**A. Demonstration of a superantigen activity in *Toxoplasma gondii*.** Tachyzoites were shown to possess molecule(s) which stimulate proliferation of and IFN- $\gamma$  synthesis by splenocytes from uninfected animals. This activity, which fulfills all of the major criteria of a superantigen, may help explain both the early induction of cell-mediated immunity by *T. gondii* and the genetics of susceptibility to disease.

**B. Role of IL-12 in innate resistance.** The induction of IL-12 was shown to be critical for the early IFN- $\gamma$ -dependent resistance of mice to acute infection but less important or non-essential for the prevention of disease reactivation.

**C. Studies on mechanisms of monokine induction by *T. gondii*.** The triggering of the monokines TNF- $\alpha$  and IL-12 by *T. gondii* was shown to be independent of macrophage Lps receptors but to involve a similar signal transduction pathway. *T. gondii* was shown to be capable of stimulating HIV replication in virally infected human monocytes or macrophages from transgenic mice containing integrated provirus.



**LABORATORY OF VIRAL DISEASES  
1994 ANNUAL REPORT  
TABLE OF CONTENTS**

<b><u>Z01 AI</u></b> <b><u>Project</u></b> <b><u>Number</u></b>	<b><u>Title</u></b>	<b><u>Page</u></b>
<b>Summary</b>		<b>19-01</b>
<b>00123-28</b>	<b>Structure and Replication of Poxvirus DNA - Merchlinsky</b>	<b>19-15</b>
<b>00126-21</b>	<b>Functional Analyses of Vaccinia Virus DNA - DeFilippes</b>	<b>19-16</b>
<b>00298-13</b>	<b>Development of Vaccinia Virus as an Expression Vector - Moss</b>	<b>19-17</b>
<b>00306-13</b>	<b>Pathogenesis of Orthopoxvirus Infection - Buller</b>	<b>19-18</b>
<b>00307-13</b>	<b>Regulation of Vaccinia Virus Gene Expression -Moss</b>	<b>19-19</b>
<b>00416-11</b>	<b>Recombinant Vaccines Against Retroviruses Associated with Leukemia and AIDS - Moss</b>	<b>19-20</b>
<b>00445-10</b>	<b>Mechanism of Viral DNA Replication - Challberg</b>	<b>19-21</b>
<b>00538-07</b>	<b>Interaction of Human Immunodeficiency Virus with CD4 Receptor - Berger</b>	<b>19-22</b>
<b>00539-07</b>	<b>Virus-Host Interactions - Moss</b>	<b>19-23</b>
<b>00541-07</b>	<b>Folding, Assembly, and Transport of Viral Glycoproteins - Yewdell</b>	<b>19-24</b>
<b>00542-07</b>	<b>Processing of Viral Proteins for T Cell Recogniton - Bennink</b>	<b>19-25</b>



## Table of Contents (Continued)

<b><u>Z01 AI</u></b> <b><u>Project</u></b> <b><u>Number</u></b>	<b><u>Title</u></b>	<b><u>Page</u></b>
00619-04	Intracellular Antibody-mediated Virus Neutralization - Yewdell	19-26
00652-03	Structure and Function of Peptide Transporters - Bennink and Yewdell	19-27
00653-03	Delivery of Antigens to the MHC class I Processing Pathway - Bennink and Yewdell	19-28
00658-03	Proteolytic Generation of Antigenic Peptides - Yewdell and Bennink	19-29
00659-03	Antigen Presentation by Human Tumors - Bennink and Yewdell	19-30
00660-03	Mechanism of Brefeldin A Action - Yewdell and Bennink	19-31
00661-03	Confocal Microscopy of Cells - Yewdell and Bennink	19-32
00690-02	Antigen Processing in Lower Eukaryotic Cells - Yewdell and Bennink	19-33
00691-02	Proteolytic Processing of Antigenic Peptides in Endoplasmic Reticulum - Bennink and Yewdell	19-34
00711-01	Characterization of a Novel Mammalian Transcription Factor Involved in Enhancer Complex Assembly - Kristie	19-35
00712-01	Regulation of RNAPII-Directed Transcription of Herpes Simplex Virus - Kristie	19-36
00713-01	Characterization of the Papillomavirus Regulatory Proteins - McBride	19-37
00714-01	Molluscum Contagiosum Virus - Moss	19-38





**LABORATORY OF VIRAL DISEASES**  
**ANNUAL REPORT, 1994**  
**SUMMARY**

The Laboratory of Viral Diseases carries out investigations on the molecular biology of viruses, the interactions of viruses with host cells, the pathogenesis of viral diseases, and host defense mechanisms. These studies are designed to increase fundamental knowledge as well as to facilitate the development of new approaches to the prevention and treatment of viral infections. Current topics of basic research include viral entry into cells, regulation of gene expression, mechanisms of DNA replication, assembly and transport of viral proteins and particles, action of viral growth factors, determinants of viral virulence, and viral targets of humoral and cellular immunity. Applied areas of investigation include development of recombinant expression vectors, candidate vaccines, and antiviral agents. These studies involve a wide range of DNA and RNA viruses including human immunodeficiency virus.

**REGULATION OF VIRAL GENE EXPRESSION.** Viruses provide advantageous systems for studying basic aspects of gene expression and can be used as expression vectors. Several different viral systems are under investigation by members of LVD.

*Herpes Simplex Virus (HSV).* HSV is used as a model system for the study of the biochemical mechanisms involved in the regulation of eukaryotic class II gene expression. Specifically, this involves the identification and characterization of the components that selectively determine the transcription-initiation rate of a given promoter. Analysis of the determinants by which these components recognize, interact, and assemble to generate active transcription complexes should provide insight into the general control of cellular gene expression. The genes of herpes simplex virus are divided into three groups. Each group is transcribed and expressed under particular conditions, representing distinct requirements for the activation of each type of promoter. This expression is dependent upon the interplay of viral and cellular factors in the recognition, assembly, and activation of specific transcription complexes. It is clear that the transcription of these genes by RNA polymerase II requires interactions between regulatory complexes assembled at distal enhancer or promoter sites and the basal factor transcription complexes that must assemble at the core promoter (TATA region). Thus, the elucidation of the components of these specific complexes and the interactions which are unique to the expression of the HSV genes can lead to the development of anti-HSV therapies.

Present studies focus upon the mammalian C1 factor, a transcription factor that is required for the assembly of the viral immediate-early gene enhancer complex. This factor was purified from mammalian cells, and cDNAs encoding the polypeptide were isolated. Analyses indicate that it is a novel transcription factor that is proteolytically processed from a large precursor protein. The continuing studies are concerned with



the distribution of this factor, the specific processing of the precursor protein, and the molecular domains which determine its interaction with the enhancer complex components. Additionally, homologous factors that are present in other animal systems are being investigated to allow for the development of a genetic system for the analysis of these proteins.

[KRISTIE]

*Poxviruses.* Unlike other DNA viruses, members of the poxvirus family replicate in the cytoplasm of the cell and encode many of the enzymes and factors needed for transcription of their genomes. Vaccinia virus, the best-studied member of this family, provides a unique system for combining biochemical and genetic approaches for investigating mechanisms of gene regulation and mRNA biosynthesis. Studies with vaccinia virus indicated that the genes are divided into three temporal classes—early, intermediate, and late—that are regulated in a cascade fashion. This past year, important new discoveries that relate to each stage of transcription were made.

*RNA polymerase-associated protein Rap94 confers promoter specificity for initiating transcription of vaccinia virus early-stage genes.* The association of a 94,000 Da viral polypeptide, called Rap94, with 30-40% of the multisubunit DNA-dependent RNA polymerase molecules purified from infectious vaccinia virus particles was established by immunoaffinity chromatography. Only the RNA polymerase molecules containing Rap94 could functionally interact with VETF, the vaccinia virus early transcription factor, to transcribe a double-stranded DNA template regulated by a viral early-stage promoter. Rap94 was required for the synthesis of short oligoribonucleotides and for the formation of stable ternary transcription complexes. These data support a model in which Rap94 confers specificity to the RNA polymerase for promoters of early-stage genes.

[AHN AND MOSS]

*Targeting of a Multicomponent Transcription Apparatus into Assembling Vaccinia Virus Particles Requires RAP94.* When expression of the vaccinia virus gene encoding RAP94 was repressed, the infectious virus yield was reduced by more than 99%. Nevertheless, intermediate- and late-stage viral gene expression and formation of ultrastructurally mature, membrane-enveloped virions occurred under the nonpermissive conditions. The RAP94-deficient particles contained the viral genome, structural proteins, early transcription factor, and certain enzymes but, unlike normal virions, had low or undetectable amounts of the viral RNA polymerase, capping enzyme/termination factor, poly(A) polymerase, DNA-dependent ATPase, RNA helicase, and topoisomerase. The presence of these viral enzymes in the cytoplasm indicated that RAP94 is required for targeting a complex of functionally related proteins involved in the biosynthesis of mRNA.

[ZHANG, AHN, AND MOSS]



*Vaccinia Virus-Encoded, Intermediate-Stage Promoter-Specific Transcription Factor Has Homology to Eukaryotic Transcription Factor SII (TFSII) and an Additional Role as a Viral RNA Polymerase Subunit.* Two new factors were isolated from vaccinia virus-infected cells that are necessary for transcription of several different intermediate-stage promoter templates but not for early- or late-stage promoter templates. VITF-1 was purified to homogeneity, and the sequences of two tryptic peptides were mapped to the fourth open reading frame within the *Hind*III E fragment (E4L) of the vaccinia virus genome, which had previously been shown to encode an RNA polymerase subunit of 30 kDa (RPO30) with homology to eukaryotic transcription elongation factor SII. Co-chromatography of VITF-1 with the E4L-derived protein was demonstrated using specific antiserum. In addition, transcriptionally active recombinant VITF-1 was made by expressing the E4L open reading frame in *Escherichia coli*. Thus, E4L encodes a multifunctional protein, serving as a RNA polymerase subunit and a stage-specific transcription factor. The stepwise binding of capping enzyme, VITF-1, and VITF-2 to a DNA/viral RNA polymerase complex was demonstrated.

[ROSALES, HARRIS, AND MOSS]

*A Cellular Factor is Required for Transcription of Vaccinia Viral Intermediate-Stage Genes.* Previous studies showed that four components from infected cells, the viral RNA polymerase and capping enzyme and two factors called vaccinia virus intermediate transcription factors (VITFs) 1 and 2, can reconstitute transcription of vaccinia virus intermediate-stage genes *in vitro*. We found that VITF-2 can be isolated from the nuclei of uninfected HeLa cells as well as from the cytoplasm of infected cells. The proteins with VITF-2 activity from uninfected and infected cells co-chromatographed and co-sedimented, suggesting that they are identical. VITF-2 activity was found in extracts of other uninfected human and monkey cells but not in nonpermissive insect cells or in conditionally permissive rabbit kidney 13 cells. VITF-2 activity was present, however, in a permissive line of rabbit kidney 13 cells that had been stably transfected with the vaccinia virus K1L host range gene. We suggest that the VITF-2 level acts as a gauge of the permissive state of the cell and thereby regulates the length of the early prereplicative phase of the infection.

[ROSALES, SUTTER, AND MOSS]

*Purification of a Prereplicative, Late-Stage-Specific Transcription Factor.* *In vivo* and *in vitro* studies have provided evidence that vaccinia virus late gene transcription factors are intermediate gene products synthesized exclusively after DNA replication. We found, however, an additional transcription factor (P3 factor) that stimulates late gene transcription but is made in the absence of viral DNA replication. P3 factor activity was not detected either in uninfected cells or in purified virions. P3 factor was stage specific, since it could not substitute for early or intermediate transcription factors. Evidence that late-stage-specific transcription factors are made both before





and after DNA replication necessitates a modification of the cascade model for vaccinia virus gene regulation.

[KOVACS, KECK, ROSALES, AND MOSS]

*Uridylate-Containing RNA Sequences Determine Specificity for Binding and Polyadenylation by the Catalytic Subunit of Vaccinia Virus Poly(A) Polymerase.*

VP55, the catalytic subunit of vaccinia virus poly(A) polymerase, has the remarkable property of adding 30 to 35 adenylates to RNA 3' ends in a rapid, processive burst before an abrupt transition to slow, non-processive adenylate addition. We demonstrated that this property results from the affinity of the enzyme for uridylate residues within the 3' 31-40 nucleotides of the RNA primer. The abrupt termination of processive polyadenylation was attributed to translocation of VP55 along the nascent poly(A) tail, which lacks uridylates for stable binding.

[GERSHON AND MOSS]

**VIRAL DNA REPLICATION.** Viruses are useful systems for analyzing the diversity of mechanisms employed in DNA replication. In addition, the virus-encoded factors provide potential targets for chemotherapy.

*Herpesviruses.* Several members of the herpesvirus family are significant human pathogens. These include herpes simplex virus (HSV), the best-studied of the family, which causes painful skin lesions and occasionally causes life-threatening encephalitis, and Epstein-Barr virus (EBV), which causes mononucleosis and is thought to be involved in some lymphocytic cancers. The study of herpesvirus DNA synthesis is useful as a model for eukaryotic DNA replication and for designing new antiviral strategies. Previous work from this laboratory demonstrated that seven HSV genes are necessary and sufficient for authentic origin-dependent DNA replication. Current efforts are directed toward studying this process with purified proteins, toward identifying required host proteins, and toward extending our studies to the lytic replication of EBV.

*Analysis of the Components of the HSV-1 Helicase-Primase Complex.* We have undertaken mutational analysis of both UL8 and UL52 in an attempt to understand the functional contribution of these polypeptides to the helicase-primase complex. One mutant in the UL52 polypeptide has been examined in detail. This mutant had equivalent levels of helicase activity compared to the wild-type UL5/UL8/52 complex but was devoid of primase activity. These data provide support for the idea that UL52 contains the catalytic site for primase activity. We have several other mutants now in which primase activity is reduced relative to wild-type but nevertheless detectable. These mutants will be useful for determining the role of conserved sequence motifs in primase catalysis.

[KLINEDINST, FABISCH, AND CHALLBERG]





*Reconstitution of DNA Synthesis in Vitro.* We have reconstituted rolling circle DNA replication *in vitro* using purified HSV replication proteins acting on a primer-template having the properties of a preformed replication fork. Our results suggest that lagging strand synthesis is very inefficient in this system. We have searched for a cellular factor that might increase the efficiency of lagging strand synthesis, to no avail. We are currently trying to solve this problem by a detailed characterization of primase enzymology.

[GOTTLIEB, KLINEDINST, AND CHALLBERG]

*Studies on the DNA Polymerase.* The HSV-1 DNA polymerase consists of a heterodimer of the UL30 (Pol; the catalytic subunit) and UL42 polypeptides. This complex is more highly processive than polymerase alone on singly-primed M13 single-stranded substrates. We have further characterized the effect of the UL42 polypeptide on a defined hairpin template-primer substrate. Gel shift and filter binding studies show that the affinity of the Pol catalytic subunit for the 3' terminus of the primer-template increases 10-fold in the presence of UL42. Our data are consistent with the idea that the increase in affinity is due to the increased binding energy generated by interaction of UL42 with DNA and with the polymerase. We are currently trying to obtain additional evidence for this model.

[GOTTLIEB AND CHALLBERG]

*Structural Studies.* To further examine the mechanism by which UL42 acts, we want to determine its structure. We have constructed a baculovirus expressing the functional domain of UL42 (about two-thirds of full length) and have scaled up our purification protocols. We are beginning crystallization trials.

[LANTZ AND CHALLBERG]

*Characterization of the Origin Binding Protein.* A number of studies have demonstrated that the HSV-1 UL9 protein, which is a homodimer in solution, binds to two high-affinity binding sites in each origin of replication. Interaction between the proteins bound at the two sites leads to the formation of a complex nucleoprotein structure. To understand this structure more fully, we have determined the stoichiometry of binding of the UL9 DNA binding domain to a single binding site. Our data unequivocally show that the protein binds to a site as a dimer.

[FIERER AND CHALLBERG]

*Epstein-Barr Virus Lytic DNA Replication.* Analysis of the structure of the EBV lytic origin and EBV genes required for lytic DNA replication suggests that, while the mechanism of DNA synthesis at an established replication fork is very similar to what occurs with HSV, the mechanism of initiation is likely to be very different. In particular, initiation depends not on a specialized viral initiator protein analogous to UL9 but on a key transcriptional transactivator protein (BZLF1). In this respect, initiation of EBV lytic DNA replication may be more similar to the initiation of cellular chromosomal DNA replication. We have begun a biochemical analysis of



EBV DNA replication. At present, we have constructed most of the key reagents necessary for these studies, which consist primarily of recombinant baculoviruses expressing the known EBV-encoded replication proteins. We are currently developing protocols for obtaining soluble enzymes.

[ZHEN AND CHALLBERG]

*Poxviruses.* Poxviruses replicate in the cytoplasm of infected cells and encode proteins needed for DNA replication.

*Identification and Characterization of the Vaccinia Virus Gene Encoding the Nicking-Joining Enzyme.* A nuclease has been detected in vaccinia virions that may have a role in the resolution of DNA concatamers. A protein that co-elutes with the nuclease activity has been purified and peptide sequences determined. The sequence corresponded to the open reading frame of a vaccinia virus gene; however, expression of this gene resulted in only low levels of nuclease. Efforts are now being made to determine whether the protein needs to be modified for optimal activity or whether a second protein is needed.

[MERCHLINSKY]

*Mutagenesis of a Conserved Region of Vaccinia Virus DNA Polymerase.* Vaccinia viruses containing codon alterations in a conserved region of the vaccinia virus DNA polymerase gene have been selected by linkage to the aphidicolin resistance mutation. Viruses with substitutions in amino acids in the conserved region have been isolated, revealing those substitutions that can be sustained by the virus *in vivo*.

[DEFILIPPES]

## VIRUS STRUCTURE, ASSEMBLY, AND CELL INTERACTIONS

*HIV.* The human CD4 molecule is able to mediate HIV infection and syncytium formation when expressed on a variety of human cell types but not when expressed on nonhuman cells. The mechanism of HIV/cell fusion and the identity of the additional human component are subjects of intense investigation, since such information may provide leads to the development of therapeutics.

*HIV Fusion Mechanisms.* Using a vaccinia-based reporter gene activation assay to measure cell fusion, evidence was obtained to refute the proposal by others that the CD26 antigen (dipeptidyl peptidase IV) is involved as an accessory cofactor in HIV-1 entry.

[BRODER, NUSSBAUM, FENG, AND BERGER]



*HIV Envelope Protein gp160 Processing.* A cell line resistant to protein toxins due to a mutation in the enzyme furin nevertheless produces functional env. This argues against the proposal by other workers that furin is essential for gp160 processing.

[BRODER, NUSSBAUM, AND BERGER]

*Therapeutic Strategies Based on the env/CD4 Interaction.* CD4-PE, a candidate AIDS therapeutic developed jointly by members of LVD, has been undergoing clinical testing. The basis for the high hepatotoxicity observed in HIV-infected patients is unclear. Experiments with mice indicated that soluble gp120 did not enhance the hepatotoxicity of CD4-PE, one possibility that had been under consideration. Analyses of new single-chain antibody-PE conjugates have been conducted. Additional collaborative projects were initiated to test the fusion-blocking activity of several anti-HIV agents that appear to act by inhibiting virus entry.

[KENNEDY, NUSSBAUM, AND BERGER]

*Paramyxoviruses.* The paramyxoviruses, especially measles, are responsible for considerable morbidity and mortality. A better understanding of the envelope glycoproteins and their interactions with cell receptors may lead to improvements in vaccines and the development of therapeutics.

*Paramyxovirus Glycoprotein/Receptor Interactions.* A vaccinia virus-based reporter gene assay was used to analyze cell fusion mediated by glycoproteins of several paramyxoviruses (SV5, measles, canine distemper). We found that, for each virus, expression of both the fusion (F) and hemagglutinin (HN or HA) glycoproteins on the same cell was required for fusion with partner cells expressing the receptor. For measles and canine distemper viruses, efficient fusion occurred with heterologous mixtures of F and HA; cell-type specificity was determined by HA. Direct evidence was obtained for functional and structural interaction between measles HA and CD46, the known receptor for measles virus.

[NUSSBAUM, BRODER, AND BERGER]

*Influenza Virus.* The major envelope glycoprotein of influenza virus, the hemagglutinin (HA), is the most extensively characterized viral envelope protein and has proven to be a useful model for studying similar proteins present on other viruses. Influenza remains a significant human pathogen, causing more mortality in the U.S.A. than any other virus. Since protection against influenza is provided nearly exclusively by the antibody response to the HA, it is essential to understand its structure to improve existing vaccines, which are only marginally effective.

*Folding, Assembly, and Transport of Viral Glycoproteins.* Monoclonal antibodies (mAbs) that distinguish between monomeric and trimeric forms of HA were used to localize the site of HA assembly in influenza virus-infected cells. This was achieved using a laser confocal scanning microscope to localize fluorescent-tagged antibodies or an electron microscope to localize colloidal gold-tagged antibodies on ultrathin



cryosections. Preliminary results suggest that trimerization occurs only after the HA has been exported from the ER. This finding is important, since cell biology dogma asserts that assembly of multimeric proteins is required for their export from the ER.

[YEWDELL AND BENNINK]

*Mechanism of Brefeldin A Action.* Brefeldin A is a fungal metabolite widely used by cell biologists and immunologists due to its unique ability to interfere with vesicular trafficking. To better understand how it works, fluorescent analogs were synthesized and localized in living or fixed cells by laser confocal scanning microscopy. The BFA conjugates localized to the endoplasmic reticulum and Golgi complex of living and fixed cells, which makes them valuable as probes to localize these organelles. Although the analogs maintained biological activity, this required cleavage by cellular esterases to release BFA from the conjugate. Nonetheless, inasmuch as the pattern localization of the BFA conjugates is unusual (and differs from the fluorescent probes contained in the conjugate), this suggests that the action of BFA is related to its specific perturbation of these membranes.

[DENG, BENNINK, AND YEWDELL]

*Poxviruses.* Vaccinia virus and other poxviruses contain multiple membranes that are assembled by mechanisms that are largely unknown.

*Effects of Rifampin on the Intracellular Distribution of Viral Protein p65.* The cytoplasmic assembly of vaccinia virus is reversibly blocked by the antibiotic rifampin, leading to the accumulation of partially membrane-delineated rifampin bodies in infected cells. Rifampin-resistant vaccinia virus mutants have point mutations in the D13L gene, which is controlled by a late promoter and expresses a 65 kDa protein, designated p65. In the presence of rifampin, p65 was found in large, cytoplasmic inclusion bodies that were distinct from rifampin bodies. The rifampin bodies themselves were labeled with p65 antibodies only after reversal of the rifampin block, predominantly on the viral crescents which rapidly formed following removal of the drug. We propose that p65 functions as an internal scaffold in the formation of viral crescents and immature virions, analogously to the matrix proteins of other viruses.

[DOMS AND MOSS]

*Orthopoxvirus Pathogenesis.* To properly evaluate the genetic elements contributing to vaccinia virus virulence in man, it is important to have well-defined model systems in which the virulence genes can be assayed. Orthopoxviruses are closely related, as indicated by DNA and antigenic analysis, but show certain distinct disease patterns depending on the virus and host animal. Because vaccinia virus replicates poorly in the available small laboratory animals, we are using a closely related virus, ectromelia virus (EV), to define the important poxvirus virulence genes.





*In Vivo Replication Mutant.* An EV ORF predicted to encode a 28 kDa protein (p28) was sequenced and found to contain a zinc finger motif that is found in a select group of proteins, some of which have transcriptional functions. An intact p28 gene was also present in CPV but not in vaccinia virus. An EV mutant lacking this ORF was shown to replicate as efficiently as parental virus in L929, primary mouse embryo, and primary mouse ovarian cells; however, this mutant virus replicated at least 100-fold less efficiently than parental virus in tested tissues of the nude, SCID, or intact mouse, spread more slowly from the primary site of inoculation to internal organs, and had an LD<sub>50</sub> that was 1000 times higher than the parental virus. p28 is essential for replication of EV in primary macrophages from the A strain of mice. EV mutant virus lacking the p28 gene expressed early functions but did not replicate DNA or express late gene functions.

[KOONIN AND BULLER]

*Nitric Oxide is a Potent Antiviral Metabolite.* Interferon gamma is important for the recovery of mice from infection with EV. The antiviral activity induced in macrophage cultures was blocked by inhibitors of nitric oxide synthase. Virus replication was also diminished in epithelial cells transfected with a cDNA encoding the inducible nitric oxide synthase or treated with organic compounds that generate nitric oxide. In mice, an inhibitor of nitric oxide synthase converted sublethal, resolving EV infections into fulminant mousepox.

[KARUPIAH AND BULLER]

## VIRAL IMMUNOLOGY

*Humoral Immunity to HIV-1.* Neutralizing antibody responses obtained with HIV-1 envelope (env) proteins are generally strain specific. A broader immune response is needed for an effective vaccine.

*Soluble Oligomeric HIV-1 env Glycoprotein Elicits Diverse Monoclonal Antibody (mAb) Reactivities.* We synthesized and purified a recombinant HIV-1 env glycoprotein, lacking the gp120/gp41 cleavage site as well as the transmembrane domain, which is secreted principally as a stable oligomer. Mice were immunized with oligomeric HIV-1 env glycoproteins to analyze the repertoire of antibody responses to the tertiary and quaternary structure of the protein. Approximately 150 hybridomas were generated, and the mAbs were assayed for reactivity by immunoprecipitation of nondenatured env protein. We found a high percentage of antibodies that recognized conformational epitopes and some that were specific for the oligomeric state. Thus, oligomeric forms of env may elicit a broader antibody response than monomeric env.

[EARL, BRODER, DOMS, AND MOSS]



## *Cellular Immunity*

*Structure and function of antigenic peptide transporters (TAPs).* The efficient presentation of antigens to the immune system requires the expression of a transporter that conveys peptides from the cytosol to the endoplasmic reticulum (ER). To analyze the structure and function of the transporters, vaccinia virus recombinants expressing the subunits of the transporters were produced. These recombinant viruses express functional TAPs that allow cells missing one or both TAP subunits to present antigens to T<sub>CD8+</sub>. Further biochemical studies using the recombinant viruses have demonstrated that both TAP1 and TAP2 bind ATP in a magnesium-dependent manner.

[RUSS, BENNINK, AND YEWDELL]

*Antigen presentation by human tumors.* It has long been hoped that tumor-specific T cells could be used to prevent and treat cancers. With this goal in mind, we created a novel type of vaccine that bypasses the normal antigen processing machinery by directly delivering antigenic peptides to the endoplasmic reticulum, the site of peptide association with major histocompatibility complex class I molecules. We have found that antigenic peptides targeted to the endoplasmic reticulum by an amino terminal signal sequence can be more immunogenic than other forms of the peptide. We are currently testing the ability of these vaccines to induce tumor-specific T cells and prevent and cure tumors in mice.

[BENNINK AND YEWDELL]

*Proteolytic generation of antigenic peptides.* One of the greatest unknowns in antigen presentation is how antigenic peptides are generated by cells. First, we have found that two genes suspected to play a role in peptide generation (LMP2 and LMP7) are not required for efficient antigen presentation. Second, to examine the role of ubiquitin-targeted proteolysis in the generation of antigenic peptides, we are using two temperature-sensitive mouse cell lines unable to grow at elevated temperatures (> 39°C) due to a mutation in a gene encoding E1-ubiquitin activating enzyme. Despite their defect in protein ubiquitination, these cells are capable of presenting viral antigens both synthesized by the cells following virus infections and delivered to the cytosol from virions following viral penetration. These results cast doubt on the necessity of protein ubiquitination for antigen processing. Third, we have examined the question of whether antigenic peptides are created during the process of protein degradation using various forms of the influenza nucleoprotein. Our findings suggest that antigenic peptides can be generated during the degradation of full-length proteins but that antigenic peptides are also generated by some other pathway, possibly involving defective products of protein synthesis.

[ANTON, GALARDY, YEWDELL, AND BENNINK]

*Delivery of antigens to the MHC class I processing pathway.* CD8<sup>+</sup> lymphocytes play an important role in host immunity to viruses and other intracellular parasites. In an effort to develop more efficient vaccines for eliciting CD8<sup>+</sup> T cells, we have produced recombinant vaccinia viruses expressing proteins that do not require



specialized antigen processing machinery. These recombinants can be more efficient in eliciting CD8<sup>+</sup> T cell responses than traditional recombinants expressing full-length gene products. In addition, these findings suggest that recombinant vaccines (including naked DNA vaccines) encoding ER-targeted peptides might be the optimal method for eliciting T<sub>CD8<sup>+</sup></sub> responses.

[BACIK, YEWDELL, AND BENNINK]

*Proteolytic processing of antigenic peptides in the endoplasmic reticulum.* A critical question in antigen processing is whether antigenic peptides are trimmed to their final length after they have been imported into the endoplasmic reticulum (ER) from the cytosol. To explore this question, we created a number of vaccinia virus recombinants expressing proteins targeted to the ER that consist of antigenic peptides surrounded by different flanking sequences. Using cells dependent on ER processing for antigen presentation, we found that the ER is only able to efficiently liberate the COOH terminal peptide of a "tandem antigenic peptide." These findings suggest that the ER has at least some endogenous peptide-trimming activity and may remove amino terminal extensions of up to seven residues by aminopeptidases following TAP-mediated translocation.

[LINK, YEWDELL, AND BENNINK]

*Class I Molecule Assembly and Trafficking.* Class I molecules of the major histocompatibility complex (MHC) bind peptides derived from cytosolic proteins and carry them to the cell surface for recognition by T cells. To study the assembly and trafficking of class I molecules, fluorescent peptides and metabolically labeled class I oligosaccharides are being used in normal and TAP-deficient mutant cells. Similar amounts of class I molecules are detected in the Golgi complexes and transported with similar kinetics to the surface of TAP-expressing and TAP-deficient cells; however, cells with TAP have two to three times more peptide-receptive class I molecules on their plasma membrane than TAP-deficient cells. This finding indicates that many of the empty class I molecules reaching the cell surface of TAP-deficient cells are defective and either cannot bind peptides or are rapidly destroyed, or both. Using confocal microscopy, we found that peptide-receptive class I molecules are concentrated in the Golgi complex of TAP-expressing cells, suggesting that dissociation of "lower" affinity peptides is probably occurring in the Golgi.

[DAY, BRUTKIEWICZ, BENNINK, AND YEWDELL]

*Antigen Processing in Lower Eukaryotic Cells.* To better define the specialized components of the antigen-processing machinery, we are attempting to reconstitute antigen processing in insect cells, which do not have a major histocompatibility complex (MHC) and would not be expected to have any of the specialized components needed to produce antigenic peptides and transport them to class I MHC molecules. We found that, following infection of mosquito cells with vaccinia viruses, gene products under the control of early-transcriptional promoters are expressed at reasonably high levels. Using recombinant vaccinia virus co-expressing class I





$\alpha$  chains with  $\beta_2$ -microglobulin, or  $\beta_2$ -microglobulin with antigenic peptides, we found that class I molecules properly fold and assemble in insect cells and maintain the capacity to bind antigenic peptides; however, the class I molecules found on the surface of mosquito cells are thermolabile, resulting in the denaturation of  $\alpha$  chain- $\beta_2$ -microglobulin heterodimers at 37°C. Co-expression of TAP with class I molecules failed to enhance the expression of thermolabile surface molecules.

[DENG, BENNINK, AND YEWDELL]

**DEVELOPMENT OF VACCINIA VIRUS AS AN EXPRESSION VECTOR.** During the past year, efforts were made to further study the host-range restricted and highly attenuated MVA strain of vaccinia virus and evaluate it as a vector.

*Protective Immunity to Influenza Virus Induced by a Recombinant Vector Derived from the MVA Strain of Vaccinia Virus.* The immunogenicity of a recombinant virus derived from MVA was investigated. Plasmid transfer vectors, which provide strong synthetic early/late promoters for the simultaneous expression of two genes as well as a transient or stable selectable marker and flanking sequences for homologous recombination with the MVA genome, were constructed. A recombinant MVA containing influenza virus hemagglutinin and nucleoprotein genes was isolated in avian cells and shown to efficiently express both proteins upon infection of human or mouse cells in which abortive replication occurs. Mice inoculated by various routes with recombinant MVA produced antibody and cytotoxic T lymphocyte responses to influenza virus proteins and were protected against a lethal influenza virus challenge as effectively as mice immunized with a recombinant derived from the replication-competent WR strain of vaccinia virus. These data suggest that live recombinant vaccines based on the MVA strain of vaccinia virus would be exceptionally safe as well as effective.

[SUTTER, WYATT, BENNINK, AND MOSS]

*Stable Expression of the Vaccinia Virus K1L Gene in Rabbit Cells Complements the Host Range Defect of a Vaccinia Virus Mutant.* Multiplication in rabbit kidney-derived RK13 cells, but not other non-permissive cells, can be restored by insertion of the vaccinia virus K1L gene into the MVA genome. During nonproductive infection of RK13 cells by MVA, transcription of representative viral early genes was revealed by Northern blotting, whereas synthesis of an intermediate mRNA and replication of viral DNA could not be detected. Despite the persistence of viral early mRNA for at least several hours, synthesis of virus-induced polypeptides occurred only during the first hour and was followed by abrupt inhibition of all protein synthesis. Transfection of RK13 cells with an eukaryotic expression plasmid that contained the K1L gene allowed MVA infection to proceed to late stages of viral protein synthesis. Moreover, RK13 cell lines that stably expressed the K1L gene were permissive for MVA as well as a K1L-deletion mutant of the WR strain of vaccinia





virus. This is the first description of the complementation of a poxvirus mutant by cells that stably express a viral gene.

[SUTTER, ROSALES, AND MOSS]

## MAJOR ADMINISTRATIVE CHANGES

*Personnel Changes.* Jack Bennink, Head of the Viral Immunology Section, was appointed Assistant Laboratory Chief. Thomas Kristie (MIT) and Alison McBride (NCI) were recruited as new tenure-track investigators. Senior Scientists Shmuel Rozenblatt (University of Tel Aviv) and Douglas Moore (USDA) are on temporary study/work assignments in LVD. Scientists who have come to LVD for postdoctoral training during this year include: Imma Barrera (University of Zurich), Randy Brutkiewicz (University of Massachusetts), Joachim Bugert (University of Heidelberg), Yu Feng (Tufts University School of Medicine), Ahlert Otteken (German Primate Center, Gottingen), and Lorena Passaralli (University of Georgia).

A number of non-tenured LVD scientists accepted positions elsewhere: Mark Buller (University of St. Louis), Michael Merchlinsky (FDA); Joseph Baldick (Princeton University), Paul Galaray (Boston University Medical School), Paul Gershon (Texas A & M University), Gunasegaran Karupiah (LIP, NIAID), Ofer Nussbaum (Shiba Hospital, Tel Aviv), Gregory Palumbo (University of Oklahoma), and Barbara Schnierle (Institute for Experimental Cancer Research, Freiburg, Germany).

*Honors, Awards, and Service.* Mark Challberg is a member of the NIH Experimental Virology Study Section and the editorial boards of *The Journal of Virology*, *Protein Expression and Purification*, and *Virus Research*. He gave an invited lecture at the International Herpesvirus Workshop.

Jack Bennink is an Associate Editor of *The Journal of Immunology* and was invited to lecture at the annual meeting of the American Society for Virology.

Jonathan Yewdell received competitive funding from the NIH Intramural AIDS Targeted Antiviral Program. He served on the NIAID/NIA Task Force on Immunology and Aging and gave invited lectures at the annual meeting of FASEB, a conference on *T Cells and Cytokines in Health and Disease* at Oxford, England, and the U.S. Japan Immunology Meeting in Hamilton, Montana.

Edward Berger received competitive funding from the NIH Intramural AIDS Targeted Antiviral Program. He served as Chairman of the NIH (Intramural): Technical Review Committee, Office of Technology Development, OD, NCI, and on the NIAID (Intramural): Technology Evaluation Advisory Committee. He gave an invited lecture and served as session chairman at the NIGMS 8th Meeting on *Structure of AIDS-Related Systems and Applications to Drug Design* and at the Workshop of



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AI 00123-28 LVD

## PERIOD COVERED

October 1, 1993 to September 30, 1994

## TITLE OF PROJECT

Structure and Replication of Poxvirus DNA

## PRINCIPAL INVESTIGATOR

M. Merchlinsky

Microbiologist

LVD, NIAID

## Others:

B. Ryan

Technician

LVD, NIAID

F. Koczo

Technician

LVD, NIAID

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Viral Diseases

## SECTION

Genetic Engineering Section

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL:

1.0

## OTHER:

2.0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

Poxviruses provide a unique system for studying the replication of DNA. Required enzymes and factors are encoded within the viral genome, and DNA synthesis and processing occur within the cytoplasmic compartment of the cell. Therefore, it has been possible to apply genetic and biochemical approaches to the study of DNA replication. Our effort has been toward ascertaining the structure and mode of replication of the poxvirus genome with particular emphasis placed on understanding the processing of the replicative intermediates—including the telomere-like hairpin structure and the enzymes involved in its replication. The replication of vaccinia virus proceeds through concatemeric intermediates, which are resolved into unit-length DNA. Mutational analysis has demonstrated that a specific *cis*-acting DNA sequence—highly conserved among poxviruses—as well as the palindromic structure of the concatemer junction, is essential for resolution, and that resolution occurred by conservative strand exchange. A model for resolution involving site-specific recombination and oriented branch migration is consistent with this data. We have identified proteins that are candidates for *trans*-acting components of telomere resolution and are characterizing their properties. A procedure has been developed for the construction of viral genomes containing large inserts of foreign DNA by ligation of subgenomic viral DNA fragments in the presence of the insert DNA. These DNA molecules are transfected into cells, where they can be packaged into viral particles and subsequently propagated as virus.

THIS PROJECT HAS BEEN TERMINATED AS OF SEPTEMBER 30, 1994.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00126-21 LVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT

Functional Analyses of Vaccinia Virus DNA

PRINCIPAL INVESTIGATOR

F. M. DeFilippes

Research Physicist

LVD, NIAID

Others:

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Viral Diseases

SECTION

Genetic Engineering Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

Previous experiments have shown that substitutions of some amino acids in a conserved region of the vaccinia virus DNA polymerase are allowed. Vaccinia virus containing the altered polymerase formed plaques similar to those of wild-type virus. To ultimately identify what aspect of DNA polymerase function was associated with the structure of this particular conserved region, an attempt was made to express the enzyme in bacteria. If modest amounts of functional enzyme can be expressed from a cloned gene, then the *in vivo* substitutions can be tested *in vitro*. Initially, about one-half of the polymerase gene, from the carboxy end, was cloned into the pET16b expression vector. This construct produced moderate amounts of a 56 kD protein after induction of bacterial cultures. The protein was insoluble, but this result was satisfactory, since it protected the protein from degradation. Vigorous isolation procedures showed that the target protein was in large inclusion bodies. To solubilize the protein, these bodies were blended at high speed in 6 M guanidine chloride and 100 mM dithiothreitol and then heated at 60°C for 60 minutes. The dissolved protein was fractionated on a sizing column in 6 M guanidine, and the 56 kD protein was collected and precipitated with ethanol. This protein was used as an antigen which will, hopefully, produce an antibody that will allow purification of polymerase from crude extracts. Also, to produce alterations of the entire DNA polymerase gene, I cloned the entire gene in M13mp19. This system produces a phage that contains a recombinant DNA with one strand of the gene. This construct allows the application of a modified form of the Eckstein mutagenesis system, in which the *in vitro* mutagenic manipulations can be completed in one day. This application depends upon the action of T5 D15 exonuclease. This enzyme has been tested and shown to degrade circular single-stranded DNA and linear and nicked circle duplex DNA. Since tests also show that closed circular DNA is unaffected, homoduplexes of altered DNA produced by the Eckstein system can be prepared from microgram amounts of the original template.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH

PROJECT NUMBER

Z01 AI 00298-13 LVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT

Development of Vaccinia Virus as an Expression Vector

PRINCIPAL INVESTIGATOR B. Moss Chief LVD, NIAID

Others: L. Wyatt Microbiologist LVD, NIAID

M. Carroll Visiting Fellow LVD, NIAID

S. Rozenblatt Visiting Scientist LVD, NIAID

COOPERATING UNITS (If any)

Parker Small, University of Florida.

LAB/BRANCH

Laboratory of Viral Diseases

SECTION

Genetic Engineering Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.6

PROFESSIONAL:

3.2

OTHER:

0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

The cytoplasmic site of gene expression and use of virally encoded enzymes is a distinguishing feature of vaccinia virus and other poxvirus vector systems that probably accounts for their consistent ability to express foreign genes derived from a variety of prokaryotic, eukaryotic, and viral sources. This feature, together with their ability to stably integrate and package large amounts of DNA without loss of infectivity, their wide host range, and the development of simple and effective methods for isolating recombinant viruses, account for their diverse use and popularity.

During the past year, we have continued to evaluate the highly attenuated MVA strain of vaccinia virus as an expression vector. Because of the inability of MVA to complete its replication cycle in human or other mammalian cells, it provides exceptional safety. A recombinant MVA that expresses the influenza virus hemagglutinin and nucleoprotein genes was constructed. Mice immunized with this recombinant virus developed a humoral and cell-mediated immune response and were protected against challenge with influenza virus. The protection was equal to or better than that achieved with a conventional replicating vaccinia virus vector.







DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00306-13 LVD

PERIOD COVERED

October 1, 1990 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pathogenesis of Orthopoxvirus Infections

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

R. M. L. Buller Expert LVD, NIAID

Others: G. Palumbo Staff Fellow LVD, NIAID  
G. Karupiah Visiting Fellow LVD, NIAID  
T. Koonina Visiting Scientist LVD, NIAID

COOPERATING UNITS (if any)

T. Fredrickson, National Cancer Institute, NIH.

LAB/BRANCH

Laboratory of Viral Diseases

SECTION

Macromolecular Biology Section

INSTITUTE AND LOCATION

NIAID, NIH Bethesda, MD 20892

TOTAL STAFF YEARS:

5

PROFESSIONAL:

4

OTHER:

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

In this project, we focused our studies on the genetic basis both of poxvirus virulence and of host resistance to virus infection. The acquired knowledge should contribute toward development of safe, effective recombinant vaccinia virus vaccines for animal and human use.

A newly identified ectromelia virus open reading frame, predicted to encode a 28 kDa protein, was shown to interact with DNA—presumably through a zinc finger motif in the carboxyl terminal region of the protein. The protein was expressed at low levels in the infected cell, and preliminary evidence suggested a nuclear site of localization. A 28 kDa mutant replicated normally in tested tissue culture cell lines but at least 100-fold less efficiently in tissues from the infected mouse, and spread more slowly from the primary site of infection to spleen and liver. The LD<sub>50</sub> of the mutant virus was at least 1000-fold lower than the parent virus. This gene appears to be an important poxvirus virulence determinant in the mousepox model. The gene is important for virus replication in the primary peritoneal macrophage of the A strain mouse.

Treatment with IFN-γ allowed mouse macrophages to restrict the replication of ectromelia, vaccinia, and herpes simplex viruses while producing nitric oxide. Inhibitors of nitric oxide synthase blocked the macrophages' antiviral activity. Virus replication was diminished in epithelial cells transfected with cDNA encoding inducible nitric oxide synthase or treated with organic compounds that generate nitric oxide. In mice, an inhibitor of nitric oxide synthase converted sublethal, resolving ectromelia virus infection into fulminant mousepox. Thus, induction of nitric oxide synthase was necessary and sufficient for a substantial antiviral effect of IFN-γ *in vitro* and *in vivo*. Synthetic nitric oxide donor compounds show broad-spectrum antiviral activity *in vitro*.

THIS PROJECT HAS BEEN TERMINATED AS OF SEPTEMBER 30, 1994



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH

PROJECT NUMBER

Z01 AI 00307-13 LVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT

Regulation of Vaccinia Virus Gene Expression

PRINCIPAL INVESTIGATOR	B. Moss	Laboratory Chief	LVD, NIAID
Others:	L. Carroll	IRTA	LVD, NIAID
	M. Casseti	Special Volunteer	LVD, NIAID
	N. Harris	Visiting Associate	LVD, NIAID
	X. Hu	IRTA	LVD, NIAID
	G. Kovacs	NRC Research Fellow	LVD, NIAID
	L. Passarelli	IRTA	LVD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Viral Diseases

SECTION

Genetic Engineering Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

7.4

PROFESSIONAL:

6.0

OTHER:

1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

Poxviruses, unlike other DNA viruses, replicate in the cytoplasm of the cell and encode many of the enzymes and factors needed for transcription of their genomes. Vaccinia virus, therefore, provides a unique system for combining biochemical and genetic approaches for investigating mechanisms of gene regulation and mRNA biosynthesis. Studies with vaccinia virus indicated that the genes are divided into three temporal classes—early, intermediate, and late—that are regulated in a cascade fashion.

This past year, important new discoveries that relate to each stage of transcription were made. The virus-encoded RNA polymerase-associated protein RAP94 was shown to confer promoter specificity for initiating transcription of vaccinia virus early stage genes. In addition, RAP94 was found to target the multicomponent transcription apparatus into assembling virus particles. Two factors for transcription of intermediate stage genes, VITF-1 and VITF-2, were isolated. VITF-1 was purified to homogeneity and identified as a viral homolog of eukaryotic transcription factor SII. VITF-2 was found to be a cellular protein that is located in the nucleus of uninfected cells. VITF-2 is the first cellular protein shown to have a direct role in vaccinia virus transcription. An additional factor, named P3, was found to be required for transcription of late genes. P3, unlike the other late transcription factors, is either an early viral protein or is induced by an early viral protein. A previously unrecognized signal, uridylyl residues within the 3' terminal end of the viral mRNA, was shown to be crucial for poly(A) polymerase to initiate poly(A) tail formation.



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH</b>		<b>PROJECT NUMBER</b> Z01 AI 00416-11 LVD
<b>PERIOD COVERED</b> October 1, 1993 to September 30, 1994		
<b>TITLE OF PROJECT</b> Recombinant Vaccines against Retrovirus Associated with Leukemia and AIDS		
<b>PRINCIPAL INVESTIGATOR</b>  Others:	B. Moss  P. Earl V. Karacostas C. Broder A. Otteken	Laboratory Chief    LVD, NIAID  Microbiologist    LVD, NIAID IRTA    LVD, NIAID IRTA    LVD, NIAID Special Volunteer    LVD, NIAID
<b>COOPERATING UNITS (if any)</b> R. Doms, University of Pennsylvania		
<b>LAB/BRANCH</b> Laboratory of Viral Diseases		
<b>SECTION</b> Genetic Engineering Section		
<b>INSTITUTE AND LOCATION</b> NIAID, NIH, Bethesda, MD 20892		
<b>TOTAL MAN-YEARS:</b> <div style="text-align: center; font-size: 1.2em;">3.1</div>	<b>PROFESSIONAL:</b> <div style="text-align: center; font-size: 1.2em;">2.7</div>	<b>OTHER:</b> <div style="text-align: center; font-size: 1.2em;">0.4</div>
<b>CHECK APPROPRIATE BOX(ES)</b> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
<b>SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)</b>  <p>Human immunodeficiency virus (HIV) is the etiological agent of acquired immunodeficiency syndrome (AIDS). At present, there is no effective vaccine against this disease, and therapeutic agents provide only limited help. The objects of this project are to characterize HIV antigens, to determine the targets of humoral and cell-mediated immunity, and to use this information to develop candidate vaccines. We have constructed recombinant vaccinia viruses containing HIV genetic information. These viruses have been used as live experimental vaccines to immunize animals, to synthesize HIV proteins in tissue culture, to make targets for cytotoxic T cells, and to study CD4-envelope interactions.</p> <p>During the past year, a novel stable, soluble oligomeric form of the HIV-1 envelope protein was genetically engineered and purified. To test the immunogenicity of the protein, a large panel of approximately 150 monoclonal antibodies was made. A large proportion of the antibodies recognized conformational epitopes on the gp120 and gp41 portions of the envelope protein. This pattern was quite different from that obtained by monomeric envelope protein.</p>		



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>PROJECT NUMBER</b> Z01 AI 00445-10 LVD	
<b>PERIOD COVERED</b> October 1, 1993 to September 30, 1994			
<b>TITLE OF PROJECT</b> Mechanisms of Viral DNA Replication			
<b>PRINCIPAL INVESTIGATOR</b>	M. D. Challberg	Section Head	LVD, NIAID
<b>Others:</b>	D. Fierer	Medical Fellow	LVD, NIAID
	J. Gottlieb	IRTA Fellow	LVD, NIAID
	D. Klinedinst	IRTA Fellow	LVD, NIAID
	W. Zhen	Visiting Associate	LVD, NIAID
<b>COOPERATING UNITS (if any)</b> Department of Pharmacology, Johns Hopkins Medical School, Baltimore, MD.			
<b>LAB/BRANCH</b> Laboratory of Viral Diseases			
<b>SECTION</b> Macromolecular Biology Section			
<b>INSTITUTE AND LOCATION</b> NIAID, NIH, Bethesda, MD 20892			
<b>TOTAL MAN-YEARS:</b> 7.0		<b>PROFESSIONAL:</b> 5.0	
		<b>OTHER:</b> 2.0	
<b>CHECK APPROPRIATE BOX(ES)</b> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews			
<b>SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)</b> <p>We are engaged in analysis of the lytic replication of two human herpesviruses: herpes simplex virus (HSV) and Epstein-Barr virus (EBV). We are studying the purified protein products of the viral genes that participate in DNA replication in lytically-infected cells, using both biochemical and molecular genetic approaches to understand the function of these polypeptides in detail.</p> <p>Our recent results regarding HSV proteins can be summarized as follows: 1) UL9, the viral protein that presumably initiates DNA replication, binds to its cognate binding site with a stoichiometry of two polypeptides per binding site; 2) The products of the HSV genes UL5, UL8, and UL52 form a three-polypeptide complex that has both helicase and primase activities. We have shown that UL52 contains the active site for primase catalysis; 3) The HSV DNA polymerase consists of a stable complex of two polypeptides: UL30, the catalytic subunit, and UL42, an accessory subunit that increases the processivity of the enzyme. We have mapped the changes that occur in the interaction between the DNA polymerase and a primer template in the presence of UL42; and 4) We have overexpressed the EBV homologs of the HSV replication proteins and are beginning biochemical analysis of these proteins.</p>			





<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>PROJECT NUMBER</b> Z01 AI 00538-07 LVD
<b>PERIOD COVERED</b> October 1, 1993 to September 30, 1994		
<b>TITLE OF PROJECT</b> Interaction of Human Immunodeficiency Virus with the CD4 Receptor		
<b>PRINCIPAL INVESTIGATOR</b>	E. Berger	Head, Molecular Structure Unit, Genetic Engineering Section  LVD, NIAID
<b>Others:</b>	C. Broder O. Nussbaum Y. Feng P. Kennedy	IRTA Fellow Visiting Associate NRC Associate Microbiologist  LVD, NIAID LVD, NIAID LVD, NIAID LVD, NIAID
<b>COOPERATING UNITS (if any)</b> Laboratory of Molecular Biology, National Cancer Institute, NIH		
<b>LAB/BRANCH</b> Laboratory of Viral Diseases		
<b>SECTION</b> X		
<b>INSTITUTE AND LOCATION</b> NIAID, NIH, Bethesda, MD 20892		
<b>TOTAL MAN-YEARS:</b> X	<b>PROFESSIONAL:</b> X	<b>OTHER:</b> X
<b>CHECK APPROPRIATE BOX(ES)</b> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
<b>SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)</b>  <p>           Infection by enveloped viruses is initiated by binding of the viral envelope glycoprotein(s) to specific receptor molecules on the target cell, followed by fusion between the viral and cellular membranes. We have been studying various aspects of viral envelope glycoprotein/receptor interactions: 1) <i>HIV env glycoprotein/CD4 interactions</i>. We have developed a novel vaccinia-based assay in which fusion between env-expressing and CD4-expressing cells leads to reporter gene activation by phage T7 RNA polymerase in the cytoplasm of the fused cells. We previously demonstrated that HIV-1 fusion is dependent on the presence of a human-specific accessory component of the CD4-expressing target cell. Our recent findings have refuted the proposal by others that the cofactor is the CD26 antigen. We have initiated approaches to identify the human-specific accessory component. Similar approaches are being applied to characterize cellular components responsible for the cell type-specific fusion specificities which we previously demonstrated for envs from T cell line-tropic vs. macrophage-tropic HIV-1 isolates. We have also examined cellular requirements for proteolytic processing of the env precursor. We found that functional env is produced in the LoVo cell line, which is known to be resistant to protein toxins due to mutation in the gene encoding furin. These results argue against the proposal of others that this protease is essential for env processing. We have continued studies of CD4-PE40, a genetically engineered hybrid toxin that we designed to selectively kill HIV-infected cells. Phase I clinical trials with CD4-PE40 are near completion; unfortunately there is no evidence of therapeutic benefit in HIV-infected adults. Other applications of this agent are still under consideration, including treatment of different categories of infected individuals (e.g., newborns of infected mothers) and <i>ex vivo</i> protocols to eliminate HIV-infected cells in the context of gene therapy and bone marrow transplantation approaches to combat AIDS. 2) <i>Paramyxovirus fusion (NEW INITIATIVE)</i>. We have initiated mechanistic studies of fusion mediated by glycoproteins of paramyxoviruses: SVS, measles virus (MV), and canine distemper virus (CDV). In all cases, cell fusion requires co-expression of the virus fusion (F) and hemagglutinin (HA or HN) glycoproteins on the surface of one cell, as well as the presence of the appropriate receptors on the other cell. For the morbillaviruses MV and CDV, fusion occurs efficiently with heterologous expression of F from one virus and H from the other; cell-type specificity is determined by H. We also obtained direct evidence for functional and structural interaction between measles H and CD46, the known MV receptor.         </p>		



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00539-07 LVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT

Virus-Host Interactions

PRINCIPAL INVESTIGATOR

B. Moss

Chief

LVD, NIAID

Others:

A. Grunhaus

IRTA Fellow

LVD, NIAID

D. Moore

Guest Researcher

LVD, NIAID

A. Ramsey-Ewing

IRTA Fellow

LVD, NIAID

R. Roper

IRTA Fellow

LVD, NIAID

E. Wolffe

IRTA Fellow

LVD, NIAID

COOPERATING UNITS (if any)

Gareth Griffiths, European Molecular Biology Laboratory, Germany

LAB/BRANCH

Laboratory of Viral Diseases

SECTION

Genetic Engineering Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

6.4

PROFESSIONAL:

6.0

OTHER:

0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

A successful infection involves: virus entry into the cell; uncoating, expression, and replication of the genome; assembly and release of infectious virus particles; and defense against specific and non-specific host immune mechanisms. Combined genetic, biochemical, electron microscopic, and immunologic approaches are being used to investigate these complex processes.

During the past year, studies have continued on the assembly of vaccinia virus. A viral protein, p65, was found to accumulate in novel inclusion bodies when the assembly of vaccinia virus was prevented by use of a specific inhibitor. Following reversal of drug treatment, the p65 protein became associated with the immature viral membranes. The p65 protein may function as a membrane scaffold to assemble the viral particle.

Vaccinia virus has at least three genes that determine the host range of the virus in tissue culture cells. The absence of the K1L gene was found to result in a block in both vaccinia virus DNA replication and transcription of the intermediate class genes in rabbit kidney cells. A rabbit cell line was constructed that stably expressed the vaccinia virus K1L gene and was permissive for viral K1L deletion mutants. This is the first description of the complementation of a poxvirus mutant by cells that stably express a viral gene.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00541-07 LVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT

Folding, Assembly, and Transport of Viral Glycoproteins

PRINCIPAL INVESTIGATOR

J. W. Yewdell

Medical Officer

LVD, NIAID

Others:

J. R. Bennink

Microbiologist

LVD, NIAID

COOPERATING UNITS (if any)

T. Bachi, Electron Microscopy Laboratory, Zurich, Switzerland; H.-P. Hauri, Biozenter, Basle, Switzerland.

LAB/BRANCH

Laboratory of Viral Diseases

SECTION

Cellular Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.2

PROFESSIONAL:

0.1

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

The improvement of current antiviral vaccines and the development of novel vaccines depends on increasing our understanding of viral attachment and fusion glycoproteins. Critical insight into understanding the antigenic structure of glycoproteins is provided by studying their interaction with monoclonal antibodies (mAbs). For a number of years, we have studied the influenza virus hemagglutinin (HA) glycoprotein. This protein serves as a model for other proteins with similar functions (e.g., HIV gp160) and, moreover, is important practically in its own right, as influenza still is a major cause of morbidity and mortality nationally and internationally.

Like many viral glycoproteins, the HA is a homo-oligomer, consisting of three identical monomeric subunits. In the past year, we continued to investigate the site of trimerization of newly synthesized HA. Our previously published findings suggested that HA trimerization occurs only after monomers are exported from the ER. This conclusion was based largely on localization studies performed with a standard immunofluorescence microscope. In the past year, we have increased the resolution of detection by using a laser confocal scanning microscope.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AI 00542-07 LVD

## PERIOD COVERED

October 1, 1993 to September 30, 1994

## TITLE OF PROJECT

Processing of Viral Proteins for T Cell Recognition

PRINCIPAL INVESTIGATOR	J. W. Yewdell	Medical Officer	LVD, NIAID
	J. R. Bennink	Microbiologist	LVD, NIAID
Others:	Patricia Day	IRTA Fellow	LVD, NIAID
	Randy Brutkiewicz	NRC Fellow	LVD, NIAID

## COOPERATING UNITS (if any)

Jan Lukso, BRB, NIAID; Ajit Varki, UCSD Cancer Center, San Diego, CA.

## LAB/BRANCH

Laboratory of Viral Diseases

## SECTION

Cellular Biology Section

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.4

## PROFESSIONAL:

2.2

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

Class I molecules of the major histocompatibility complex (MHC) consist of a highly polymorphic heavy chain complexed to  $\beta_2$  microglobulin ( $\beta_2m$ ). Class I molecules are expressed on virtually all cell types. Their sole function is to bind antigens and present them to T cell-bearing CD8 molecules. CD8<sup>+</sup> T cells play a critical role in eradicating intracellular pathogens and tumors. They can also contribute to immunopathology, being involved in organ rejection and autoimmune diseases. There has been rapid progress in understanding the physical nature of the antigen-class I complex and in how antigens are generated and become associated with class I molecules in cells. Peptides of 8 to 15 residues produced from a cytosolic pool of proteins by cytosolic proteases are translocated into the endoplasmic reticulum (ER) by a MHC-encoded transporter complex known as TAP. Once in the ER, peptides (possibly after further trimming by peptidases) bind to class I molecules associated with TAP and are transported to the cell surface.

In the past year, we have continued our studies of the assembly and trafficking of MHC class I molecules and have made progress on two fronts: 1) we developed fluorescent derivatives of antigenic peptides and used these probes to make several novel findings about the intracellular trafficking of class I molecules and antigenic peptides, and 2) we have studied the intracellular trafficking of class I molecules using [<sup>3</sup>H]-labeled saccharides to label class I-associated N-linked oligosaccharides.





<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>PROJECT NUMBER</b> Z01 AI 00619-04 LVD
<b>PERIOD COVERED</b> October 1, 1993 to September 30, 1994		
<b>TITLE OF PROJECT</b> Intracellular Antibody-Mediated Virus Neutralization		
<b>PRINCIPAL INVESTIGATOR</b> J.R. Bennink J. W. Yewdell	Microbiologist Medical Officer	LVD, NIAID LVD, NIAID
<b>COOPERATING UNITS (if any)</b>  		
<b>LAB/BRANCH</b> Laboratory of Viral Diseases		
<b>SECTION</b> Viral Immunology Section		
<b>INSTITUTE AND LOCATION</b> NIAID, NIH, Bethesda, MD 20892		
<b>TOTAL MAN-YEARS:</b> 0.2	<b>PROFESSIONAL:</b> 0.1	<b>OTHER:</b> 0.1
<b>CHECK APPROPRIATE BOX(ES)</b> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
<b>SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)</b> <p>           One of the greatest hurdles in creating vaccines for a number of viral pathogens is the antigenic variability of viral coat proteins. The rapid mutation rate of a number of viruses (most notably influenza virus and HIV) allows the virus to escape the neutralizing antibody response induced by antiviral vaccines. The internal proteins of the virus, on the other hand, are generally highly conserved and have not evolved to rapidly alter their antigenicity. Antibody responses to these internal proteins, while a consistent feature of immune responses to viruses, almost always fail to influence viral infectivity, since the antibodies do not have access to their target antigens, which are located either inside the virus or inside the virus-infected cells. If, however, antibodies are introduced into the cytosol of cells, they do have the ability to prevent viral infection. Recent advances in understanding antibody folding indicate that antibodies can, under some circumstances, properly fold and bind antigen when they are expressed in the cytosol by removing their amino terminal ER insertion sequences. Therefore, cells or, eventually, transgenic animals expressing such cytosolic antibodies should be resistant to virus infection. Using electroporation to introduce antibodies into the cytosol, we identified antibodies able to block infection of cells with influenza A or B viruses. Our ultimate goal is to clone the genes encoding these antibodies and to express them in the cytosol of tissue culture cells and, eventually, transgenic animals, thus creating animals resistant to influenza virus infection.         </p>		



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00652-03 LVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT

Structure and Function of Peptide Transporters

PRINCIPAL INVESTIGATOR

J.W. Yewdell

Medical Officer

LVD, NIAID

J.R. Bennink

Microbiologist

LVD, NIAID

Others:

Gustav Russ

Visiting Associate

LVD, NIAID

COOPERATING UNITS (If any)

T. Bachi, University of Zurich, Switzerland; P. Cresswell, Yale University, New Haven, CT.

LAB/BRANCH

Laboratory of Viral Diseases

SECTION

Cellular Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.4

PROFESSIONAL:

1.2

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

CD8<sup>+</sup> T cells play an important role in controlling virus infections. CD8<sup>+</sup> T cells recognize peptides of 8 to 10 residues derived from viral proteins located in the cytosol of virus-infected cells. These peptides are recognized in a complex with class I molecules encoded by the major histocompatibility complex (MHC). In the past three years, it was discovered that the MHC also encodes two molecules, termed TAP1 and TAP2, that combine in a 1:1 ratio to create a complex that specifically transports peptides from the cytosol into the endoplasmic reticulum (ER). Human TAP genes display at least some limited polymorphism. The existence of these peptide pumps and their polymorphism raises a number of important questions: Where in the cells are the pumps located? Do the pumps influence the types of peptides presented by class I molecules? Are the pumps tethered to the proteases that produce antigenic peptides in the cytosol? How do the pumps work? Are there individuals with immune deficiencies based on mutations in the TAP genes? Can cells transport peptides *via* other mechanisms?

To help characterize the structure and function of the TAP genes, we have created recombinant vaccinia viruses (rVV) that express either TAP1 (VV-TAP1<sub>h</sub>), TAP2 (VV-TAP2<sub>h</sub>), or TAP1 and TAP2 (VV-TAP[1&2]<sub>h</sub>). We have demonstrated that each of the rVVs express functional subunits or, in the case of the VV-TAP[1&2]<sub>h</sub>, a functional transporter. Using this panel of rVVs, we have studied the assembly, intracellular location, and biochemical properties of TAP1 and TAP2.



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>PROJECT NUMBER</b> Z01 AI 00653-03 LVD
<b>PERIOD COVERED</b> October 1, 1993 to September 30, 1994		
<b>TITLE OF PROJECT</b> Delivery of Antigens to the MHC Class I Processing Pathway		
<b>PRINCIPAL INVESTIGATOR</b>	J.R. Bennink J. W. Yewdell  Others: I. Bacik	Microbiologist Medical Officer Visiting Fellow  LVD, NIAID LVD, NIAID LVD, NIAID
<b>COOPERATING UNITS (if any)</b> B. Murphy, LID, NIAID, NIH; N. Restifo, Surgery Branch, NCI, NIH; S. Tevethia, Hershey Medical Center, Hershey, PA; V. Nussenweige, New York University, New York, NY.		
<b>LAB/BRANCH</b> Laboratory of Viral Diseases		
<b>SECTION</b> Viral Immunology Section		
<b>INSTITUTE AND LOCATION</b> NIAID, NIH, Bethesda, MD 20892		
<b>TOTAL MAN-YEARS:</b> <div style="text-align: center;">0.9</div>	<b>PROFESSIONAL:</b> <div style="text-align: center;">0.7</div>	<b>OTHER:</b> <div style="text-align: center;">0.2</div>
<b>CHECK APPROPRIATE BOX(ES)</b> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
<b>SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)</b>  CD8 <sup>+</sup> lymphocytes play an important role in host immunity to viruses and other intracellular parasites. Antiviral CD8 <sup>+</sup> T cells recognize MHC class I molecules bound to peptides derived from a cytosolic pool of viral proteins. The induction of antiviral CD8 <sup>+</sup> T cell responses is potentially limited by the rates at which peptides are generated from full-length gene products and the rate at which peptides are transported into the ER by TAP, the MHC-encoded peptide transporter. In an effort to develop more efficient vaccines for eliciting CD8 <sup>+</sup> T cells, we created a number of recombinant vaccinia viruses that express antigenic peptides in the absence of flanking residues or with an amino terminal extension that targets the peptides to the endoplasmic reticulum. Testing of these recombinants indicates that these recombinants more efficiently elicit CD8 <sup>+</sup> T cell responses than traditional recombinants expressing full-length gene products.		



PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Proteolytic Generation of Antigenic Peptides**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

J. R. Bennink	Microbiologist	LVD, NIAID
J. W. Yewdell	Medical Officer (Research)	LVD, NIAID

Others: Igor Bacik	Visiting Fellow	LVD, NIAID
Luis Anton	Visiting Fellow	LVD, NIAID
Paul Galaray	Hughes Scholar	LVD, NIAID

COOPERATING UNITS (if any)

T. Spies, Dana Farber Cancer Institute, Boston, MA.

LAB/BRANCH

Laboratory of Viral Diseases

SECTION

Viral Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.9

PROFESSIONAL:

1.7

OTHER:

1.2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

CD8-expressing T cells play a critical role in eradicating intracellular parasites such as viruses. CD8<sup>+</sup> T cells recognize MHC class I molecules in a complex with peptides of 8 to 10 residues derived from viral proteins located in the cytosol. There is tremendous interest in the mechanism by which peptides are generated. There is indirect evidence that implicates proteasomes in the generation of antigenic peptides. Proteasomes are abundant macromolecular structures present in the cytosol and nucleus in cells, and have multiple protease activities. They are thought to be responsible for energy-dependent proteolysis in which ubiquitin plays a prominent role in targeting proteins for destruction. Although it is believed that at least some proteolysis occurs in the cytosol, it is uncertain whether the ultimate determinants are generated in the cytosol or whether additional trimming occurs following transport from the cytosol.

We have initiated several approaches to assess the site and nature of proteolytic mechanisms utilized in the generation of antigenic peptides. First, we have studied antigen processing in cells defective in their capacity to ubiquitinate proteins. Second, we have examined the relationship between metabolic stability of a protein and its efficiency as a source of antigenic peptides. Third, we examined the requirement for MHC-encoded proteasome subunits in antigen processing.





<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>PROJECT NUMBER</b> Z01 AI 00659-03 LVD
<b>PERIOD COVERED</b> October 1, 1993 to September 30, 1994		
<b>TITLE OF PROJECT</b> Antigen Presentation by Human Tumors		
<b>PRINCIPAL INVESTIGATOR</b> J. R. Bennink J. W. Yewdell	Microbiologist Medical Officer	LVD, NIAID LVD, NIAID
<b>COOPERATING UNITS (if any)</b> N. Restifo and S. Rosenberg, Surgery Branch, National Cancer Institute, NIH.		
<b>LAB/BRANCH</b> Laboratory of Viral Diseases		
<b>SECTION</b> Viral Immunology Section		
<b>INSTITUTE AND LOCATION</b> NIAID, NIH, Bethesda, MD 20892		
<b>TOTAL MAN-YEARS:</b> 0.4	<b>PROFESSIONAL:</b> 0.2	<b>OTHER:</b> 0.2
<b>CHECK APPROPRIATE BOX(ES)</b> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
<b>SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)</b>  <p>There is a considerable body of evidence that tumor-specific CD8<sup>+</sup> T lymphocytes can be elicited in tumor-bearing individuals and can mediate a significant antitumor effect. In most cases, however, tumor-specific CD8 T cells do not arise naturally. We are investigating the possibility that the poor immunogenicity of some tumors is due to limitations in the abilities of cancer cells to present their tumor-specific antigens to the immune system.</p> <p>In the past few years, there has been tremendous progress in our understanding of how cells present antigens for recognition by CD8 T cells. It is now clear that presentation of antigen results from a chain of events, including protein breakdown into peptides of 8 to 10 amino acids, transport of peptides from the cytosol into the endoplasmic reticulum, binding of peptides to a special peptide-presenting molecule, and delivery of the peptide-complexed presenting molecule to the cell surface. To test the hypothesis that limitations in tumor antigen breakdown or transport of the antigen into the endoplasmic reticulum limit the immunogenicity of T cells, we have created novel vaccines in which antigenic peptide generation and transport occurs independently of the normal antigen processing machinery. We are currently testing whether such vaccines are able to elicit tumor-specific CD8 T cells and can protect animals against a challenge with tumor cells or cure animals with existing tumors. We have created a vaccine for possible use in patients afflicted with melanoma.</p>		



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00660-03 LVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT

Mechanism of Brefeldin A Action

PRINCIPAL INVESTIGATOR	J. W. Yewdell	Medical Officer	LVD, NIAID
	J. R. Bennink	Microbiologist	LVD, NIAID
Others:	Y. Deng	Visiting Fellow	LVD, NIAID

COOPERATING UNITS (if any)

R. Haugland and H.C. Kang, Molecular Probes, Eugene, OR.

LAB/BRANCH

Laboratory of Viral Diseases

SECTION

Cellular Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.90	0.7	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

Brefeldin A (BFA) is a fungal metabolite with a broad range of biological activities, including antifungal and antitumor activities. In the past few years, there has been tremendous interest in its effects on cells, since it has been found to have a unique ability to interfere with vesicular trafficking in cells. Among its effects, it disperses Golgi complex, returning at least some of its components to the endoplasmic reticulum (ER). BFA also prevents newly synthesized proteins destined for the cell surface from leaving the ER. This property is responsible for its ability to completely block the presentation of viral antigens to cytotoxic T lymphocytes. To better understand the mechanism of BFA action, we conjugated it to two fluorescent dyes and studied its intracellular localization. Both conjugates maintained their biological activity and both specifically localized to the ER and Golgi complex in live or aldehyde-fixed cells. The selective partitioning of conjugated BFA into intracellular membranes is probably due to its interaction with lipids as it is abolished by detergent extraction of lipids. These conjugates are the first dyes that bind the ER and Golgi complex without binding other prominent membrane bound compartments and should prove useful as probes for these organelles in living cells. The interaction of BFA with the ER and Golgi complex membranes may be essential to its effects on vesicular trafficking.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00661-03 LVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT

Confocal Microscopy of Cells

PRINCIPAL INVESTIGATOR

J. W. Yewdell

Medical Officer

LVD, NIAID

J. R. Bennink

Microbiologist

LVD, NIAID

COOPERATING UNITS (if any)

K. Kelly, LP, DCBD, National Cancer Institute; L. Staudt, MET, National Cancer Institute, NIH

LAB/BRANCH

Laboratory of Viral Diseases

SECTION

Cellular Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.4

PROFESSIONAL:

0.2

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

Recent advances in optics and electronics have culminated in the development of the laser confocal scanning microscope (LCSM). The LCSM provides unparalleled resolution for localizing fluorescent probes in cells. In combination with advanced—yet relatively inexpensive and compact—computers, it is relatively easy to produce 3-dimensional reconstructions of fluorescently labeled structures in cells. We have been working with a BioRad MRC 600 LCSM with its controlling computer. To produce 3-D reconstructions of data sets obtained with the LCSM, we use VoxelView software on a Silicon Graphics Iris Indigo computer. We have used this system for a wide variety of applications, including determining the subcellular distribution of newly defined proteins and following the intracellular trafficking of newly synthesized membrane proteins.



Z01 AI 00690-02 LVD

**PERIOD COVERED****TITLE OF PROJECT**

PRINCIPAL INVESTIGATOR

LVD, NIAID

Others:

LVD, NLAIID

**COOPERATING UNITS (If any)**

LAB/BRANCH

## SECTION

INSTITUTE AND LOCATION

**TOTAL MAN-YEARS:**

0.9

---

**PROFESSIONAL:**

0.7

**OTHER:**

0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

**SUMMARY OF WORK** (Use standard unreduced type. Do not exceed the space provided)

19-33





<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>PROJECT NUMBER</b> Z01 AI 00691-02 LVD	
<b>PERIOD COVERED</b> October 1, 1993 to September 30, 1994			
<b>TITLE OF PROJECT</b> Proteolytic Processing of Antigenic Peptides in the Endoplasmic Reticulum			
<b>PRINCIPAL INVESTIGATOR</b>	J. R. Bennink J. W. Yewdell	Microbiologist Medical Officer	LVD, NIAID LVD, NIAID
<b>Others:</b>	Heidi Link	IRTA Fellow	LVD, NIAID
<b>COOPERATING UNITS (if any)</b>			
<b>LAB/BRANCH</b> Laboratory of Viral Diseases			
<b>SECTION</b> Viral Immunology Section			
<b>INSTITUTE AND LOCATION</b> NIAID, NIH, Bethesda, MD 20892			
<b>TOTAL MAN-YEARS:</b> <div style="text-align: center;">1.9</div>		<b>PROFESSIONAL:</b> <div style="text-align: center;">1.7</div>	
		<b>OTHER:</b> <div style="text-align: center;">0.2</div>	
<b>CHECK APPROPRIATE BOX(ES)</b> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews			
<b>SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)</b>  <p>           CD8<sup>+</sup> T cells recognize class I molecules of the major histocompatibility complex (MHC) bearing peptides of 8 to 10 residues derived from cytosolic proteins. These cells are a bulwark of host defenses to infectious agents and tumors, and it is critical to understand how antigenic peptides are generated by cells if we are to improve existing vaccines and develop new vaccines and treatments for infectious and neoplastic diseases. Very little is known about how cells produce antigenic peptides from proteins. While it is clear that proteolytic production of peptides begins in the cytosol, it is uncertain to what extent trimming occurs after peptides are translocated into the endoplasmic reticulum (ER). Previous results from our laboratory indicate that trimming of peptides in the ER can occur under the special circumstances of expressing an exotic secretory protease in antigen-presenting cells. To extend these findings, we have used a novel strategy to characterize the endogenous proteolytic capacity of the ER and its relevance to antigen processing. Our findings demonstrate that ER proteases, most likely aminopeptidases, are able to liberate antigenic peptides from longer precursors. In conjunction with recent findings from other laboratories, our results suggest a model in which peptides from cytosolic proteins are transported into the ER with amino terminal extensions that are removed by an aminopeptidase residing in the ER.         </p>			



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00711-01 LVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT

Characterization of a Novel Mammalian Transcription Factor Involved in Enhancer Complex Assembly

PRINCIPAL INVESTIGATOR

T. M. Kristie

Senior Staff Fellow

LVD, NIAID

Others:

COOPERATING UNITS (If any)

J. Pomerantz and P. Sharp, Center for Cancer Research-MIT, Cambridge, MA; S. Parent, Merck Research Laboratories, Rahway, NJ

LAB/BRANCH

Laboratory of Viral Diseases

SECTION

Molecular Genetics Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.8

PROFESSIONAL:

0.8

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

Expression of the immediate early genes of herpes simplex virus I requires the assembly of a multiprotein RNAPII enhancer complex consisting of several viral and cellular transcription factors. This assembly therefore provides a model for the analysis of the components involved in the specific activation of gene expression. The present study focuses upon the characterization of these factors, their interactions, and the mechanisms by which they specifically induce the expression of the viral genes. Although several of these protein factors have been purified and characterized, little is known concerning the mammalian C1 factor, a large protein complex which interacts with the viral transactivator and is required for the enhancer complex assembly. Therefore, this factor was purified from mammalian cells, and cDNAs encoding the polypeptide were isolated. Analysis of this factor indicate that it is a novel transcription factor which is specifically proteolytically processed from a large precursor protein. The continuing studies focus upon questions concerning the tissue distribution of this factor, the specific processing of the precursor protein, and the molecular domains which determine its interaction with the enhancer complex components. Additionally, homologous factors which are present in other animal systems are being investigated to allow for the genetic analysis of these proteins.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 AI 00712-01 LVD
PERIOD COVERED October 1, 1993 to September 30, 1994		
TITLE OF PROJECT Regulation of RNAPII-Directed Transcription of Herpes Simplex Virus		
PRINCIPAL INVESTIGATOR	T. M. Kristie	Senior Staff Fellow LVD, NIAID
Others:		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Viral Diseases		
SECTION Molecular Genetics Section		
INSTITUTE AND LOCATION NIAID, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 0.2	PROFESSIONAL: 0.2	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided) <p>Transcription of eukaryotic genes by RNAPII requires the assembly of multiple protein factors into a transcription initiation complex. In addition, the regulation of this process with respect to the selective activation of specific genes and the rate at which these genes are transcribed are often dependent upon interactions of protein regulatory factors within this complex. Herpes simplex virus is used as a model system for the analysis of the genetic sequences and protein factors required for the regulated transcription of mammalian genes by RNAPII. In the course of a viral infection, three classes of genes are transcribed in a distinct temporally regulated manner. This study is involved in the development of an <i>in vitro</i> transcription system which reproduces the regulatory requirements of each viral gene class. Such a system will allow for the analysis of the basic and specific protein factors which selectively activate a particular gene class as well as elucidation of the biochemical mechanisms involved in this process.</p>		



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 AI 00713-01 LVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT

Characterization of the Papillomavirus Regulatory Proteins

PRINCIPAL INVESTIGATOR	A. A. McBride	Investigator	LVD, NIAID
Others:	J. L. Brokaw	IRTA Fellow	LVD, NIAID
	M. H. Skiadopoulos	IRTA Fellow	LVD, NIAID

COOPERATING UNITS (If any)

LAB/BRANCH

Laboratory of Viral Diseases

SECTION

Molecular Genetics Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.6

PROFESSIONAL:

1.6

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

Papillomavirus transcription and DNA replication are regulated, at least in part, by the products of the viral E1 and E2 open reading frames. Structural and functional analyses of the E1 and E2 proteins have been carried out to provide some insight into the mechanisms by which these proteins regulate the viral life cycle.

The E1 and E2 proteins form a complex that binds co-operatively to the viral origin of replication. It has been shown that a region of the E1 protein between residues 162 and 378 is important for origin-specific DNA binding. A large C-terminal domain of the E1 protein is required for co-operative DNA binding and for interaction with the E2 protein.

The N-terminal 200 amino acids of the E2 protein forms a multifunctional domain important for transcription and replication. In an attempt to separate these properties and to understand the mechanisms by which the E2 proteins function, an extensive mutational analysis of this domain has been carried out. These studies have identified E2 residues important for transcriptional regulation and DNA replication.

A short peptide sequence that is responsible for nuclear targeting of the E2 proteins has been identified. This signal is contained within the region of the E2 DNA binding domain that forms a recognition helix for sequence-specific DNA-binding.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00714-01 LVD

PERIOD COVERED

January 1994 to November 30, 1994

TITLE OF PROJECT

Molluscum Contagiosum Virus

PRINCIPAL INVESTIGATOR

B. Moss

Laboratory Chief

LVD, NIAID

Others:

J. Bugert

Special Volunteer

LVD, NIAID

T. Koonina

Visiting Associate

LVD, NIAID

COOPERATING UNITS (if any)

Gholamreza Darai, University of Heidelberg, Germany; Eugene Koonin, National Library of Medicine, NIH.

LAB/BRANCH

Laboratory of Viral Diseases

SECTION

Genetic Engineering Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.9

PROFESSIONAL:

1.5

OTHER:

0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

Molluscum contagiosum virus is a human poxvirus that causes lesions that can persist for months to years in the skin of healthy or immunodeficient individuals, particularly children. Infection with this virus is increasing and has become a troublesome complication for adults with AIDS. At present, there is no specific preventive or treatment for this disease. All attempts to culture this virus have failed, and little is known about it. The object of this project is to analyze the structure of the viral genome and determine its mode of expression and replication. Cloned DNA fragments representing the entire genome of molluscum contagiosum virus have been obtained, and sequencing has been initiated.



LABORATORY OF MALARIA RESEARCH  
1994 Annual Report  
Table of Contents

Z01 AI

Project Number

Page

Summary		14-1
00108-23	Studies on the Immunobiology of Malaria - Miller	14-4
00208-14	The Isolation and Characterization of Plasmodial Genes - McCutchan	14-5
00240-13	Antigenic Analysis of Sexual Stages of Malaria Parasites - Kaslow	14-6
00241-13	Identification of Receptors for Merozoite Invasion of Erythrocytes - Miller	14-7
00248-13	Genetics and Physiology of Vector Capacity in Anopheline Mosquitoes - Gwadz	14-8
00483-09	Genetics of <i>P. falciparum</i> - Wellems	14-9
00629-03	Population Biology of <i>Anopheles gambiae</i> in Africa - Gwadz	14-10



### ADMINISTRATIVE AND ORGANIZATIONAL EVENTS

The Laboratory of Malaria Research is conducting studies on a wide range of topics relating to the malaria parasite and its mosquito vectors with emphasis on parasite/red cell receptors, molecular genetics of resistance to anti-malarial drugs, evaluation of various antigens as potential vaccine candidates, and methods for producing and delivering these antigens.

The Laboratory's involvement with the Malaria Research and Training Center of the National School of Medicine and Pharmacy in Bamako, Mali, continues. In the last year, the research facilities at the Center have been significantly improved, and a number of major research efforts have been initiated. A ground station at the Center now links Mali through Healthnet to the worldwide Internet system. Newly installed CD-ROM systems provide full bibliographic search capabilities for the biomedical literature, and a procedure for receiving full text copies of journal articles from the National Library of Medicine is now in place. Research at the MRTC includes studies on the rapid detection of drug-resistant malaria using molecular probes, the etiology and control of severe and complicated malaria, preparations for testing a transmission-blocking vaccine, characterization of the genetic makeup of vector populations, and strategies for malaria control based on the use of impregnated curtains.

This has been an important period of research on receptors that has identified a new domain that is involved in binding of parasites to erythrocytes (C. Chitnis, K.L. Sim and L.H. Miller). This cysteine-rich domain is involved in binding to glycophorin A (*P. falciparum*) and to the Duffy blood group system (*P. vivax*). It has also been a period that has led to the identification of the erythrocyte receptors for both *P. falciparum* and *P. vivax*.

G. Ward has made progress in a most difficult area of invasion related to signaling and host membrane changes when the parasite interacts with the host cell membrane. I. Tardieux with G. Ward have developed an actin-binding column to isolate proteins of the parasite that may be involved in the moving junction. They have so far identified the HSP-70 as a component and are awaiting sequence data to identify other components.

D. Hudson and L. H. Miller have found that an oxidoreductase is a component of the junction. It is a member of a gene family found from prokaryotes to humans, but unlike any other member of this family, it has an asymmetrical charge that may be involved in binding to skeletal components of the junction. The proof that the malarial protein has antioxidant activity and why it should be located in the junction remains to be explored.



S. Kumar, L. H. Miller and D. Kaslow have been successful in protecting monkeys from challenge by a highly virulent strain of *P. falciparum*. The vaccine contains a component of the C-terminus of the major malaria surface protein.

J. Tian, L.H. Miller and S. Kumar have been successful in developing a recombinant *Salmonella* that will lead to protection against a virulent strain of *P. yoelii*. It should be possible now to immunize against an entire library of *P. falciparum* to identify new protective antigens.

S. Kumar, A. Yadava, L.H. Miller and A. Sher have found that IL-12 accelerates immunity to *P. yoelii*. The mechanism is now being explored.

In the Molecular Biology Section, T. McCutchan and G. McConkey have isolated a new class of drug-resistant mutants of *P. berghei*. One of the mutants is resistant to pyrimethamine while the second is resistant to 5-fluoroorotic acid which interferes with de novo pyrimidine synthesis; studies are underway to define these mutations at the molecular level.

In the Genetics and Pharmacology Section, X. Su and T. Wellems continued to map the 200 kb segment of chromosome 7 associated with chloroquine resistance. Using cDNA libraries with yeast artificial chromosomes (YACs), they have now sequenced over 60kb within the segment and have identified several genes, none of which appear to govern chloroquine resistance. A detailed microsatellite map across the 200 kb segment has been developed to identify crossover events, thereby reducing the size of the region in which the gene encoding chloroquine resistance is found.

Y. Wu, working on transfection in malaria parasites, has preliminary results which indicate that a chloramphenicol-acetyl transferase reporter has been expressed in intraerythrocytic *P. falciparum*. Efforts are being directed at optimization of signals and the production of stably-transfected parasites.

D. Peterson has been characterizing a gene family whose members contain a sequence motif found in several erythrocyte-binding proteins. These genes constitute a highly diverse, multicopy family present in all *P. falciparum* isolates examined. One of these genes has been linked in a genetic cross to an enhanced invasion phenotype.

Working with scientists in Mali, C. Plowe has improved techniques for rapid genetic analysis of *P. falciparum* carrying mutations for resistance to pyrimethamine and cycloguanil. The tests can now be performed on field collected blood stored on filter paper, an advance which will greatly facilitate collection and storage of blood samples.

D. Kaslow in the Molecular Vaccine Section is making final preparations for clinical trials of a transmission-blocking malaria vaccine based on Pfs 25, the dominant surface





antigen of the early mosquito midgut forms of the malaria parasite *P. falciparum*. A yeast produced sub-unit vaccine linked to alum will soon be tested in volunteers at the NIH Clinical Center. Preliminary studies have been initiated at the MRTC to prepare for field tests of the this vaccine candidate in Mali.

P. Duffy and D. Kaslow have cloned and sequenced Pfs 28, the dominant surface protein of the late mosquito midgut forms of *P. falciparum*. The recombinant protein has been expressed in yeast and elicits transmission-blocking antibodies in mice. A yeast-produced chimeric protein incorporating sequences from both Pfs 25 and Pfs 28 is being developed.

K. Williamson has found that antisera generated against *E. coli* produced recombinant Pfs230, expressed in gametocytes of *P. falciparum*, reduces the infectivity of parasites to mosquitoes by 71.8% - 89.9%, indicating that this region of the protein is a good candidate for a malaria transmission-blocking vaccine.

T. Templeton has produced a high resolution cDNA library that is being mass sequenced to identify clones containing secretory signal sequences involved in the process of fertilization of the malaria parasite in the mosquito gut.

M. Shahabuddin has shown that trypsin like serine proteins present in the mosquito midgut activate the malaria parasite-produced chitinase critical to ookinete penetration. Serine protease inhibitors completely block malaria development in the mosquito by interfering with normal passage of the parasite through the peritrophic matrix.

In the Molecular Entomology Section, K. Vernick, L. Miller and M. Aikawa described the process of ookinete lysis in a selected refractory line of the vector, *Anopheles gambiae*.

M. Valencia, L. Miller and P. Mazur, working on techniques for cryopreservation of anopheline embryos, have developed a method for permeabilization of mosquito embryos which permits uptake of cryoprotectants without affecting eventual survival.

E. Mialhe, E. Saraiva, and L. Miller have developed a biolistic system capable of introducing DNA into anopheline mosquito embryos. Transposons which can facilitate DNA integration in the mosquito genome are now being evaluated.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00108-23 LMR

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT

Studies on the Immunobiology of Malaria

PRINCIPAL INVESTIGATOR	I. H. Miller	Chief	LMR, NIAID
	D. Kaslow	Section Head	LMR, NIAID
	S. Kumar	Visiting Associate	LMR, NIAID
Others:	T. Jing-Hui	Visiting Fellow	LMR, NIAID
	A. Yadava	Visiting Fellow	LMR, NIAID

COOPERATING UNITS (If any)

J.A. Berzofsky, Sr. Scientist, Metabolism Branch, NCI; A. Agar, U. of Miami; W.E. Collins, CDC, Atlanta, GA; G. Milon, Pasteur; E. Classen, Holland; W. Jacobs and Barry Bloom, Albert Einstein Univ.

LAB/BRANCH

Laboratory of Malaria Research

SECTION

Malaria Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

Immunity to asexual erythrocytic parasites in certain rodent malarias is dependent on cell mediated mechanisms that are dependent on CD4<sup>+</sup> T cells and the spleen and are independent of antibody. Such immunity can be induced by Salmonella and malarial antigens. The present studies are designed to identify those proteins and T cell epitopes that will lead to protection. In addition, we are developing studies to test constructs in Salmonella and BCG that will lead to protection against Plasmodium falciparum in Aotus monkeys.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00208-14 LMR

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Isolation and Characterization of Plasmodial Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Thomas F. McCutchan	Microbiologist	
Others:	G. McConkey	Senior Staff Fellow	LMR, NIAID
	M. Sullivan	Technician	LMR, NIAID
	J. Li	Visiting Associate	LMR, NIAID
	M. J. Rogers	Sr. Staff Fellow	LMR, NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Malaria Research

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

2.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have continued our work on the developmentally regulated transcription of *Plasmodium* ribosomal rRNA genes. We have defined the pattern of expression of the different units during parasite development in the mosquito. We have found that up-regulation of one unit relates to the differentiation of the sporozoite as a unit within the oocyst. We have also discovered a previously undescribed rDNA unit that is expressed only during early oocyst development in the mosquito. In separate studies, we have continued our work with attenuated lines of human and rodent malarial. Auxotrophic lines of parasites have been cloned and their developmental characteristics studied. Auxotrophic mutants of both species of parasite have been defined by their dependency on either exogenous pyrimidines or para-aminobenzoic acid. These should prove useful in the study of both the biochemistry of parasites and the development of immunity to parasites.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00240-13 LMR

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Antigenic Analysis of Sexual Stages of Malaria Parasites

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

D. C. Kaslow Senior Staff Fellow LMR, NIAID  
Others: K. Williamson, Guest Researcher, LMR, NIAID; M. Shahabuddin, Visiting Fellow, LMR, NIAID; M. Fried, Visiting Fellow, LMR, NIAID; T. Templeton, IRTA Fellow, LMR, NIAID; P. Duffy, Guest Researcher, LMR, NIAID; D. Keister, Biologist, LMR, NIAID; O. Muratova, Special Volunteer, LMR, NIAID; R. Hearn, Entomologist, LMR, NIAID; M. Criscio, Student Trainee Biol., LMR, NIAID

COOPERATING UNITS (If any)

(see attached)

LAB/BRANCH

Laboratory of Malaria Research

SECTION

Molecular Vaccine Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD

TOTAL STAFF YEARS:

8.6

PROFESSIONAL:

6.3

OTHER:

2.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

One to three million children in Africa will die of malaria this year. To control malaria, several vaccine approaches are being developed, one of which is against the sexual stages (aka a transmission-blocking vaccine). The genes encoding four potential transmission-blocking target antigens (Pfs25, Pfs28, Pfs40, and Pfs230) of transmission-blocking antibodies have now been cloned in our laboratory, and a fifth, a parasite-produced chitinase, and a sixth, a mosquito-produced protease, have been identified. Of the four target antigens that have been cloned, all have been expressed in one or more recombinant expression systems, but only rPfs25 and rPfs28 have induced transmission-blocking antibodies in laboratory animals. Our immediate goals are to 1) test in humans the safety, immunogenicity, and efficacy of a rPfs25 subunit vaccine and design a means of testing the efficacy of such a transmission-blocking vaccine in the field, 2) improve expression of the rPfs28 that induces blocking antibodies and test various combinations of rPfs25 and rPfs28 in a cocktail vaccine suitable for use in humans to determine if the combination elicits longer lasting or higher titer transmission-blocking antibodies, 3) determine the role, if any, that the calcium-binding Pfs40 plays in sexual development and ascertain if Pfs40 is a target of transmission-blocking antibodies, 4) improve the expression of rPfs230 so that it induces antibodies equivalent to those of transmission-blocking mAbs to Pfs230, 5) isolate and express the genes encoding the parasite-produced chitinase and mosquito-produced proteases, and 6) isolate analogous genes to the five parasite proteins, if they exist, from *P. vivax*. Our more long-term goals include identifying new target antigens on sexual stage parasites, and defining the molecular mechanisms involved in fertilization of malarial parasites.





## (Cooperating Units Cont.)

J. Coligan, Branch Chief, BRB, NIAID; B. Moss, Lab Chief, LVD, NIAID; J. Shiloach, Unit Chief, LCBD, NIDDK; Immunex Corporation, Seattle, WA (V. Price); Catholic University, Nijmegen, The Netherlands (J. Schoenmakers and R. Sauerwein); Pasteur Institut, Paris, France (G. Langsley); Scripps Clin. Res. Fnd., La Jolla, CA (A. Satterthwait, E. Stura, A. Kang); CDC, Atlanta, GA (B. Collins, A. Lal, T. Ruebush); US Army (Walter Reed Army Institute of Research, Washington, D.C. -R. Ballou and J. Sadoff); University of Edinburgh, Edinburgh, Scotland (E. Riley); Case Western Reserve School of Medicine, Cleveland, OH (M. Aikawa); Hahnemann University, Philadelphia, PA (A. Vaidya); University of Hawaii, Honolulu, HI (G. Hui and S. Chang); Queensland Institute of Medical Research, Brisbane, Australia (A. Saul); Virogenetics Corp., Troy, NY (E. Paoletti), SmithKline and Beecham, King of Prussia, PA (M. Gross).



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00241-13 LMR

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT

Identification of Receptors for Merozoite Invasion of Erythrocytes

PRINCIPAL INVESTIGATOR

L. H. Miller

Chief

LMR, NIAID

Others:

D. Hudson-Taylor Microbiologist

LMR, NIAID

C. Chitnis

Visiting Fellow

LMR, NIAID

J. Smith

Visiting Fellow

LMR, NIAID

G. Ward

Visiting Associate

LMR, NIAID

I. Tardieux

Visiting Fellow

LMR, NIAID

COOPERATING UNITS (if any)

F. Klotz and J.D. Haynes, WRAIR; M. Aikawa, Case Western Reserve University; T. Hadley, University of Louisville; E. Sass-Toby, NICHD; Horuk, Genentech; K.L. Sim, Entremed; O. Poga and Ashok, New York Blood Center; E. Koonin, National Library of (See below)

LAB/BRANCH

Laboratory of Malaria Research

SECTION

Malaria Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

5.5

PROFESSIONAL:

5.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

(Cooperating units cont.)

Medicine; E. Sass-Toby, NICHD; L. Tilney, U. Pennsylvania; G. Langsley, Inst. Pasteur; Y. Raviv, NIDDK.

The merozoite interacts in a receptor-specific manner with the erythrocyte surface and is the stage upon which immunity may work to block invasion. Thus, merozoite surface components are of interest because of their role in erythrocyte recognition and as antigens for induction of protective immunity. We are identifying *P. knowlesi*, *P. vivax* and *P. falciparum* receptors for attachment to monkey and human erythrocytes. The components in the junction and the signaling after merozoites make contact with erythrocytes is also under study.



**DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT**

**PROJECT NUMBER**

Z01 AI 00248-13 LMR

**PERIOD COVERED**

October 1, 1993 to September 30, 1994

**TITLE OF PROJECT**

Genetics and Physiology of Vector Capacity in Anopheline Mosquitoes

**PRINCIPAL INVESTIGATOR**

L. H. Miller	Laboratory Chief	LMR, NIAID
R. W. Gwadz	Section Head	LMR, NIAID
K. D. Vernick	Senior Staff Fellow	LMR, NIAID

Others: D. C. Kaslow, Medical Staff Fellow, LMR, NIAID; M. Shahabuddin, Visiting Fellow, LMR, NIAID; E. Mialhe, Guest Researcher, LMR, NIAID; M. P. Valencia, Visiting Fellow, LMR, NIAID; E. Saravia, Visiting Fellow, LMR, NIAID; C. Barreau, Visiting Fellow, LMR, NIAID; D. Seeley, Supervisory Entomologist, LMR, NIAID; A. Laughinghouse, Research Entomologist, LMR, NIAID; P. Pimenta, Guest Researcher, LPD, NIAID

**COOPERATING UNITS (If any)**

National School of Medicine, Bamako, Mali (Dr. Y. Toure); Oak Ridge National Laboratory (Dr. P. Mazur); Case Western Reserve University (Dr. M. Aikawa)

**LAB/BRANCH**

Laboratory of Malaria Research

**SECTION**

Molecular Entomology Section

**INSTITUTE AND LOCATION**

NIAID, NIH, Bethesda, MD 20892

**TOTAL STAFF-YEARS:**

8.1

**PROFESSIONAL:**

5.2

**OTHER:**

2.9

**CHECK APPROPRIATE BOX(ES)**

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

**SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided)**

Other Cooperating Units: Biological Laboratories, Harvard Univ. and the European Molecular Biology Laboratory, Heidelberg, Germany (Drs. F. Kafatos and B. Zheng); Department of Molecular Biology and Biochemistry, University of California-Irvine (Dr. A. James).

The biology of anopheline mosquitoes is being studied in relation to the capacity of these vectors to transmit malaria. Genetic, molecular, biochemical and immunological studies are seeking to describe mechanisms in the mosquito which enhance or retard development of the malaria parasite with emphasis on the behavior of ookinetes, oocysts and sporozoites.

To facilitate these studies: 1) methods are being improved for cloning and transposing genes into mosquito germ lines, and 2) systems are being evaluated for the cryopreservation of mosquito embryos to increase our capacity to store genetic material.

The ability to identify, clone, and transpose genes which regulate mechanisms which render mosquitoes refractory to parasite development should facilitate development of control strategies based on the introduction into the field of mosquito populations incapable of transmitting malaria.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00483-09 LMR

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetics studies of *P. falciparum*

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Thomas E. Wellems	Section Head	LMR, NIAID
Others:	David Peterson	Senior Staff Fellow	LMR, NIAID
	Xin-zhuan Su	Senior Staff Fellow	LMR, NIAID
	Yimin Wu	Research Fellow	LMR, NIAID
	Françoise Guinet	Research Fellow	LMR, NIAID
	Christopher Plowe	Medical Staff Fellow	LMR, NIAID
	Kathleen Creedon	Guest Researcher	FDA
	Laura Kirkman	Technician	LMR, NIAID

COOPERATING UNITS (if any)

Wellcome Trust Research Institute, Nairobi (W. Watkins); Hahnemann University, Philadelphia (A. Vaidya); Sloan Kettering Institute, New York (J. Ravetch); Catholic University, Washington, D.C. (P. Rathod); (see attached page)

LAB/BRANCH

Laboratory of Malaria Research

SECTION

Genetics and Pharmacology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 10892

TOTAL STAFF YEARS:

7.2

PROFESSIONAL:

7.0

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Molecular genetic approaches are being used to study determinants of drug response in *Plasmodium falciparum*, to isolate genes involved in the interaction of this parasite with host red blood cells, to characterize a defect of parasite gametogenesis and to identify factors that affect severe malaria.

Genetic basis of chloroquine resistance: Chloroquine resistance in a *P. falciparum* cross is controlled by a determinant within a 200 kb segment of the parasite's 7th chromosome. We are isolating transcribed sequences from this segment and evaluating them as candidate genes. Field studies of drug resistance: Assays for dihydrofolate reductase-thymidylate synthase point mutations that confer pyrimethamine and proguanil resistance have been improved and are being applied to epidemiological studies in Kenya and Mali. Other drug targets: *P. falciparum* S-adenosylhomocysteine hydrolase has been expressed in *E. coli* and is being purified for kinetic and functional comparisons against the mammalian enzyme. Transfection of parasite erythrocytic stages: Preliminary results indicate that a chloramphenicol-acetyl transferase reporter has been expressed in intraerythrocytic *P. falciparum*. Efforts are being directed at optimization of signals and the production of stably-transfected parasites. Mobile and variable gene families within the parasite genome: Two families of repetitive coding elements (*gfh*, *eb1*) that are highly variant in their organization among different parasites have been identified by positional cloning of transcripts from chromosome 7. The *gfh* elements have several properties suggestive of retroposon-like sequences, while *eb1* members have motifs found in some genes known to be involved in red cell invasion. One of the *eb1* genes maps to a subtelomeric region of chromosome 13 previously implicated in the efficiency of invasion. A defect in *P. falciparum* gametogenesis: Poor differentiation of male gametes in one parent of a genetic cross has been found to be a quantitatively inherited trait that maps by linkage analysis to chromosome 12. A physical map of this chromosome is being developed with a view toward isolation of candidate genes from the linkage region. Hemoglobinopathies and the malaria hypothesis: A field study of the occurrence of hemoglobin C and malaria is being initiated in Mali to determine whether this hemoglobinopathy confers protection against severe forms of the disease.





(Cooperating units Cont.)

NIMH, NIH (R. Aksamit); ENMRP, Bamako (D. Diallo, A. Djimde, O. Doumbo); University of Toronto, Toronto (K. Kain); Institut Pasteur, Paris (A. Scherf)



**DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE**  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

**PROJECT NUMBER**  
 Z01 AI 00629-03 LMR

**PERIOD COVERED**

October 1, 1993 to September 30, 1994

**TITLE OF PROJECT**

Population Biology of *Anopheles gambiae* in Africa

**PRINCIPAL INVESTIGATOR**

R. W. Gwadz	Section Head	LMR, NIAID
R. K. Sakai	Senior Scientist	LMR, NIAID

**Others:**

K. D. Vernick	Senior Staff Fellow	LMR, NIAID
G. C. Lanzaro	Senior Staff Fellow	LMR, NIAID

**COOPERATING UNITS (if any)**

National School of Medicine, Bamako, Mali (Dr. Y. Toure); Inst. of Parasitology, Univ. of Rome (Dr. V. Petrarca); Univ. of Maryland Biotechnology Inst. (Dr. R. Colwell); J.G. Estrada-Franco, Guest Researcher, LMR, NIAID

**LAB/BRANCH**

Laboratory of Malaria Research

**SECTION**

International Research Section

**INSTITUTE AND LOCATION**

NIAID, NIH, Bethesda, MD 20892

**TOTAL STAFF-YEARS:**

3.4

**PROFESSIONAL:**

2.8

**OTHER:**

0.6

**CHECK APPROPRIATE BOX(ES)**

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

**SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)**

Other Cooperating Units: Biological Laboratories, Harvard University and the European Molecular Biology Laboratory, Heidelberg, Germany (Dr. F. Kafatos and B. Zheng)

*Anopheles gambiae* is the primary mosquito vector of malaria in Africa. As a prelude to the evaluation of strategies for malaria control based on the concept of replacement of vector populations with mosquitoes unable to transmit the malaria parasite, we are examining, in depth, the genetic and biologic structure of vector populations in a series of distinct ecological zones in Mali (West Africa). To facilitate these studies, we are using the tools of molecular genetics directed at microsatellite polymorphisms, restriction length polymorphisms (RFLPs), and ribosomal and mitochondrial DNA. Field studies of vector populations using these techniques will be conducted at the Malaria Research and Training Center in Bamako, Mali in collaboration with staff of the National School of Medicine and Pharmacy of Mali.



**LABORATORY OF INTRACELLULAR PARASITES**  
**Rocky Mountain Laboratories**  
**Hamilton, Montana**  
**1994 Annual Report**  
**Table of Contents**

<b>ZO1 AI</b>		
<b>Project Number</b>		<b>Page</b>
Summary		8-1
00216-14	Molecular Chlamydial Vaccine Development -- Caldwell	8-10
00519-07	Immunopathogenesis of Chlamydial Infections -- Morrison	8-11
00552-06	Immunopathobiology of Bacterial Toxins -- Cieplak	8-12
00553-05	Molecular Biology and Immunology of Pathogenic Campylobacter spp. -- Cieplak	8-13
00567-04	Cellular Biology of Host-Parasite Interactions -- Hackstadt	8-14
00631-03	Developmental Biology of Intracellular Parasites -- Hackstadt	8-15
00672-02	Mucosal Immunity to Chlamydial Infection - Caldwell	8-16
00693-02	Pathogenic Mechanisms of <i>Mycobacterium</i> <i>tuberculosis</i> - Barry	8-17



Annual Report  
Laboratory of Intracellular Parasites  
Rocky Mountain Laboratories  
Hamilton, Montana  
National Institute of Allergy and Infectious Diseases  
October 1, 1993 to September 30, 1994

Research Highlights

Scientists in the Laboratory of Intracellular Parasites (LICP) study infectious diseases caused by obligate and facultative intracellular bacterial pathogens. These include *Chlamydiae*, *Mycobacteria*, *Rickettsiae*, and bacterial enteropathogens. Molecular, biologic, and immunologic approaches are used to delineate the structure/function relationship of parasites in their interaction with host cells and to delineate immune mechanisms that function in the development of acquired immunity to infection. The role of chlamydial histone H1 homologs in DNA structure and gene expression during chlamydial differentiation are being studied. Trafficking of biosynthetic precursors through cellular organelles to the chlamydial vesicle and modification of that vesicle by pathogen derived proteins are current areas of research. Molecular biology and modern chemistry are being used to elucidate the details of biosynthetic pathways leading to the synthesis of mycobacterial mycolic acids. The results of this work are being used to design new anti-mycobacterial drugs that can be targeted at the biosynthesis of mycolic acids. Studies on enteropathogens focus on the identification of virulence genes of *Campylobacter jejuni* involved in invasiveness and response to intracellular infection. *Escherichia coli* heat-labile enterotoxin and cholera toxin are being investigated by directed mutagenesis and other biochemical techniques to identify structures that function in the toxic and immunomodulatory properties of these proteins. Animal models are being used to study acquired immunity to several bacterial pathogens with specific emphasis on delineating the role of T-cells in protective and pathologic immune responses. The results of this work are being used to formulate rational strategies for the development of subunit and recombinant vaccines.

**Immunology of Intracellular Parasites Section:** The Immunology Section is under the direction of Dr. Harlan Caldwell. There are two major research themes within the Immunology Section; (i) development of animal models for the study of protective and pathologic immune responses to chlamydial infection, and (ii) the development of a safe and efficacious chlamydial vaccine. A murine animal of chlamydial genital tract infection has been developed and knock out (KO) mice are being used to define immune mechanisms that function in acquired immunity to chlamydial infection. The results of this work show that MHC class II and CD4<sup>+</sup> deficient strains of mice fail to resolve chlamydial genital tract infection whereas  $\beta_2$ -microglobulin and MHC class I KO mice resolved infection similarly to C57BL/6 parent control mice. Serologic analysis showed that class II and CD4<sup>+</sup> KO mice produced far less antibodies of all isotypes and failed to produce local secretory IgA. Additionally, chlamydial specific DTH was not





demonstrated in class II and CD4<sup>+</sup> deficient mice. These findings indicate that CD4<sup>+</sup> T-cells are essential for the development of protective immunity to chlamydial genital tract infection and implicate chlamydial specific sIgA and DTH as important effector mechanisms in recovery from chlamydial genital tract infection. Future work will focus on identification of specific CD4<sup>+</sup> T-cell subsets (Th1, Th2) that function in protective immunity and characterization of those chlamydial antigens that protective T-cells recognize.

Projects involving vaccine development have been ongoing since the inception of LICP. This work has focused on the design of synthetic oligopeptides immunogens corresponding to key T-helper and neutralizing B-cell epitopes of the chlamydial major outer membrane protein (MOMP) and the development of infectious vectors expressing MOMP neutralizing epitopes that are capable of targeting chlamydial specific neutralizing antibodies at the oculogenital mucosae. Accomplishments in this area includes the molecular mapping of MOMP T-helper and neutralizing epitopes, synthesizing these epitopes as co-linear peptide immunogens, and defining the peptides immunogenicity and protective efficacy in animal models of chlamydial infection. Prototype peptide vaccines have been shown to target the production of functional anti-chlamydial neutralizing antibodies and to have a high potential for wide MHC recognition. Efficacy studies in mice and non-human primates indicate that parenteral immunization with the peptide vaccine provides partial protection against chlamydial infection of the eye and genital tract. These findings indicate that a preventative chlamydial vaccine might be possible and have led to research to pursue more effective vaccine formulations; specifically those that are capable of targeting local sIgA responses against chlamydiae. Towards this end, recombinant vaccine strains of poliovirus have been constructed that express key MOMP neutralizing epitopes as poliovirus VP1 fusions. Poliovirus/MOMP hybrids have been shown to grow nearly as well as parent virus and to target the production of high titered anti-chlamydial neutralizing antibodies. Recombinant poliovirus/MOMP hybrids are being used as infectious vaccine vectors to target chlamydial specific sIgA responses and efficacy studies are planned in the future using a non-human primate model of chlamydial infection.

**Host-Parasite Interactions Unit:** The Host-Parasite Interactions Unit is under the direction of Dr. Ted Hackstadt. There are two major research areas within The Host-Parasite Interaction Section; i) the role of histone-like proteins in the control of chromatin structure and gene expression by *Chlamydia trachomatis* and *Coxiella burnetii*, and ii) the cell biology of the interaction of obligate intracellular parasites with the host. Modification of DNA structure by histone-like proteins appears to be a central regulatory mechanism governing the complex life cycle of *Chlamydia trachomatis*. Expression of Hc1 in *E. coli* results in a compaction of the chromatin that ultrastructurally resembles the nucleoid reorganization which occurs late in the chlamydial developmental cycle and causes a global termination of transcription, translation, and replication at concentrations equivalent to that of chlamydial elementary bodies. Since we suspect that any regulatory effects of chlamydial Hc1 are likely a consequence of its modification of DNA structure, our emphasis is upon detailed biochemical analysis of DNA-protein and protein-protein interactions in conferring higher-order chromatin structure. Longer term goals will address some obvious remaining questions such as the environmental signals which trigger



histone synthesis and/or aggregation and how the histones may be released from DNA at the initial stages of the developmental cycle. Understanding of the environmental signals which trigger histone activity is severely hampered by a lack of information regarding the physical and nutritional parameters within the vesicle in which chlamydiae undergo their life cycle. There are many fundamental questions concerning the nature of the intracellular compartment in which chlamydiae replicate. We have made two significant advances in our understanding of the chlamydial inclusion. A novel experimental approach has identified secreted chlamydial polypeptides that are inserted into the inclusion membrane. The inclusion itself seems to interact with the Golgi apparatus as demonstrated by direct trafficking of a sphingolipid probe through the Golgi to the cell wall of the chlamydiae themselves. A further implication is that the inclusion membrane is derived from the Golgi. Collectively, these results suggest a Golgi-derived vesicle that is modified by the insertion of chlamydial proteins.

**Molecular Pathogenesis Unit:** The Molecular Pathogenesis Unit is under the direction of Dr. Witold Cieplak, Jr. The recent publication of the X-ray crystal structure of LT, refined to 1.95 Å resolution, has allowed the investigation of the potential involvement of various residues in a predicted NAD-binding cleft. The refined crystal structure has shown that arginine 7, a residue section members previously showed to be involved in enzymatic activity, lies within the predicted NAD-binding site. More recently, section members have altered histidine 44, serine 61, alanine 69, and tryptophan 127 using multiple isoionic or isosteric substitutions. These residues are predicted to lie within or be vicinal to the substructure or cleft presumed to be the NAD-binding site. While virtually all substitutions at these residues are capable of dramatically reducing enzymatic activity, retention of gross conformation, as judged by sensitivity to limited trypsinolysis, is dependent upon the type of substitution introduced. Mutations that have been judged to result in reductions of enzymatic activity but no detectable alterations in overall conformations are being introduced into the gene for the holotoxin to assess their effects on toxin assembly and cytotoxic activity. Members of the Section have begun analyses of potential residues involved in the NAD-binding site using photolabeling techniques. These studies have been facilitated by the development of an FPLC purification scheme that results in preparations of recombinant A subunits and analogs of greater than 95% purity. Direct photolabeling with [carbonyl-<sup>14</sup>C]-NAD and ultraviolet irradiation has been used and occurs at a level of 20-30 mol %. The labeling is saturable, time dependent, and results in the specific inactivation of enzyme activity. Labeled peptides are being isolated and will be subjected to microsequence analysis to determine the actual site of photolabeling; preliminary analyses using proteolytic fragmentation of the labeled A subunit indicates that the vast majority of the label lies in a peptide containing two glutamic acid residues (110 and 112) that are thought to be critical to catalytic activity. In a collaborative effort with Dr. Boyd Haley (University of Kentucky), members of the section are also employing photoactivatable derivatives of NAD (2-azido-NAD) to map the NAD-binding site. To date, effective photolabeling of recombinant LT A subunit has been achieved at low temperature. Interestingly, both direct photolabeling and photolabeling using 2-azido-NAD have revealed that a co-factor, termed ADP-ribosylation factor (ARF), that is required for the activity of LT and CT appears to bind to the toxins at or near the NAD binding site. These findings will have important implications in establishing the precise nature of the interactions between the



toxins, ARF, and the target G proteins. Previous studies have shown that *C. jejuni* alters its protein synthetic profile during interaction with epithelial cells in culture. These observations prompted section members to investigate the nature of the proteins that are novel synthesized or enhance during the processes of binding and internalization. To facilitate characterization of the genes, several expression libraries of *C. jejuni* genomic DNA were created and section members have been able to maintain genomic fragments in a stable fashion through use of a methyl-mismatch repair deficient strain of *E. coli* as the host. Genomic clones have been screened for preferential reactivity using antisera generated against bacteria grown in the presence and absence of epithelial cells. To date, several clones reacting preferentially with antisera prepared against bacteria cultivated with epithelial cells have been identified. One clone and derivatives have been completely sequenced and they appear to encode the homolog of the widespread bacterial DNA-binding protein termed HU. The preferential immunoreactivity of this protein is puzzling since it is normally quite abundant during bacterial growth. It is noteworthy, however, that the HU gene is only the fifth *C. jejuni* gene to be cloned and sequenced, and as such provides additional information regarding the characteristics of promoters and other regulatory elements in this organism. Additional immunoreactive clones have been identified and are in the process of being characterized. (Cieplak, Konkell, Grant)

**Mycobacterium Unit:** The Mycobacterium Unit is under the direction of Dr. Clifton Barry, III. Understanding the biosynthesis and function of complex cell-wall molecules and using this knowledge to enable the development of novel chemotherapeutic treatments for mycobacterial diseases is the major focus of the Tuberculosis Research Unit. We have utilized the ability to transform mycobacteria to localize at least one biosynthetic gene directly involved in mycolic acid biosynthesis. Pathogenic species of mycobacteria (including *M. leprae*, *M. tuberculosis*, and *M. avium*) uniformly modify their major mycolic acids at two positions by enzymatically transforming a double bond into a cyclopropane ring while saprophytic strains such as *M. smegmatis* do not. *M. smegmatis* containing a recombinant cosmid library from *M. tuberculosis* was screened for cyclopropanation of mycolic acids by purifying and examining them on a thin-layer chromatography system which selectively retards components containing *cis* double bonds (the cyclopropane precursor). Two of 600 cosmid-containing clones appeared to possess the pathogenic structural type of mycolic acid. This modified mycolic acid was purified and its structure was found to be a not naturally-occurring hybrid of the normal *M. smegmatis* major mycolate with the *M. tuberculosis*-like cyclopropane in the distal position. Isolation of the DNA from these two clones has revealed that they were overlapping, non-identical cosmids containing a common 7.2kb BamHI fragment of which we have currently sequenced about 6kb. In this region we have discovered an open reading frame of 864 nucleotides which codes for a protein of 288 amino acids. This protein displays a high degree of homology to the *E. coli* cyclopropane fatty acid synthase - the only characterized enzyme which catalyzes the formation of a cyclopropane ring. The *cma* gene alone is sufficient to convert *M. smegmatis* mycolates to the cyclopropanated type. In addition to the cyclopropanating enzyme, we have discovered two additional genes which appear to encode enzymes related to lipid biosynthetic pathways by homology to known systems. Together these two ORFs could be components of a unique kind of Type II fatty acid synthase involved in pre-meromycolate extension or modification. These three genes offer strong evidence that we are within an operon encoding multiple enzyme





activities related to mycolic acid biosynthesis.

A related ongoing project illustrates the potential of this information. In the biosynthesis of mycolic acids a 24 carbon fatty acid is specifically desaturated and then extended and cyclopropanated. We designed and synthesized a series of compounds which contain a sulfur atom at the position which is ultimately cyclopropanated (actually we synthesized a homologous series of thioethers with the sulfur in each of four different positions since the exact position of methyl group addition was unknown). When tested *in vitro* against growing *Mycobacterium tuberculosis* these compounds displayed a potent anti-bacterial activity with MIC<sub>50</sub>s of the best of these approaching that of isoniazid (1µg/ml of 4-Thiatetracosanoate). Importantly, *M. smegmatis*, which does not cyclopropanate its mycolic acids, is unaffected by these drugs. This work has enabled us to define a portion of the pathway which was unclear from previous published work and illustrates the potential of designing antimycobacterial drugs based upon a detailed knowledge of the biosynthetic pathway of mycolic acids.





## ADMINISTRATIVE REPORT

The LICP is divided into four sections: (i) the Host-Parasite Interactions Unit (ii) the Immunology of Intracellular Parasites Section, (iii) the Molecular Pathogenesis Unit, and (iv) the Mycobacterium Unit. The Host-Parasite Section is headed by Dr. Ted Hackstadt, the Immunology Section by Dr. Harlan Caldwell, the Molecular Pathogenesis Unit by Dr. Witold Cieplak and the Mycobacterium Unit by Dr. Clifton E. Barry, III. Scientists working in the Host-Parasite Section include: Dr. Paul Policastro (Senior Staff Fellow), Dr. Richard Garzon (IRTA), Dr. Robert Heinzen (IRTA), Dr. Daniel D. Rockey (IRTA), Dr. Marci Scidmore (IRTA) and Scientists working in the Immunology Section include: Dr. Richard Morrison (Research Microbiologist), Dr. Hua Su (Visiting Associate), Dr. Scott Manning (Staff Fellow), Dr. Todd Cotter (IRTA). Scientists working in the Molecular Pathogenesis Unit are Dr. Christopher Grant (Visiting Associate), and Dr. Michael Konkel (IRTA). Scientists working in the Mycobacterium Unit are Dr. Yuan Ying (Visiting Associate) and Katie George (IRTA). The total FTE and non-FTE scientific slots allotted to LICP are occupied. Summer students working in LICP include: Brian Chesebro (Pre-IRTA), Kevin Gray (Pre-IRTA) and Melissa Reeves (Clerical). The following people were invited by LICP staff to present seminars: Dr. Richard Garzon, Medical College of Virginia, Richmond, VA, Dr. Jeff Actor, IRTA/NIAID, Bethesda, MD, Dr. Olivia Steele-Mortimer, European Biology Laboratory, Heidelberg, Germany, Dr. Katie George, University of Chicago School of Medicine, Chicago, IL., Dr. You-Xun Zhang, Maxwell Finland Laboratory for Infectious Diseases, Boston City Hospital, Boston, MA., Dr. Khisimuzi Mdluli, University of Victoria, and Dr. Dana Wolle, University of Indiana.



## HONORS AND AWARDS

### Journal Editorial Boards:

H. Caldwell - Infection and Immunity  
W. Cieplak - Infection and Immunity

### Ad Hoc Reviewers:

H. Caldwell - Cell  
Journal of Bacteriology  
Journal of Infectious Diseases  
Journal of Immunology  
Proceedings of the National Academy of Sciences  
Microbiol Pathogenesis

W. Cieplak - Journal of Bacteriology

T. Hackstadt - Journal of Bacteriology  
Molecular Microbiology  
Gene  
Journal of Infectious Diseases  
Trends in Microbiology

R. Morrison - Journal of Clinical Investigation  
Infection and Immunity  
Journal Infectious Diseases  
Journal of Immunology  
Microbial Pathogenesis

### Professional Posts:

H. Caldwell - Faculty affiliate, Division of Biological Sciences (Microbiology),  
University of Montana, Missoula, Montana.

R. Morrison - Faculty affiliate, Division of Biological Sciences (Microbiology),  
University of Montana, Missoula, Montana.

### Elected Post:

T. Hackstadt - Councilor-at-large, American Society for Rickettsia and Rickettsial  
Diseases.

R. Morrison - Chair, Division of General Medical Microbiology, American Society for  
Microbiology, 1994-95.



### Invited Lectures and Participation in Meetings and Symposia:

- C. Barry - Seminar, Structure and Chemistry Lecture Series, The Scripps Research Institute, La Jolla, CA.  
Seminar, Colorado State University, Ft. Collins, Colorado.  
Seminar, American Society for Microbiology/Canadian Society for Microbiology, Joint Meeting, Victoria, B.C.  
American Society for Microbiology, Las Vegas, Nevada.  
Seminar, PathoGenesis Corporation, Seattle, Washington
- H. Caldwell- United Biomedical, Hauppauge, New York  
University of Goteborg, Goteborg, Sweden  
NIAID, Vaccine Board, Bethesda, Maryland  
Centers for Disease Control, Atlanta, Georgia  
American Society for Microbiology, Las Vegas, Nevada  
8th International Chlamydial Meeting, Paris, France
- T. Cotter - American Society for Microbiology, Las Vegas, Nevada  
8th International Chlamydial Meeting, Paris, France
- T. Hackstadt - Seminar, University of N. Carolina, Chapel Hill, North Carolina  
Northwest Branch of ASM/Western Branch of the Canadian Society of Microbiologists, Victoria, B.C., Canada  
University of Montana, Missoula, Montana  
American Society for Microbiology, Las Vegas, Nevada  
8th International Chlamydial Meeting, Paris, France  
Gordon Conference on Bacterial Toxins and Pathogenesis, Andover, New Hampshire  
ASRRD, St. Simons Island, Georgia
- R. Heinzen - American Society for Microbiology, Las Vegas, Nevada  
ASRRD, St. Simons Island, Georgia
- S. Manning - American Society for Microbiology, Atlanta, Georgia
- R. Morrison - Seminar, University of South Dakota, Brookings, South Dakota  
Seminar, University of Alabama, Birmingham, Alabama  
American Society for Microbiology, Las Vegas, Nevada
- P. Policastro- ASRRD, St. Simons Island, Georgia
- H. Su - 8th International Chlamydial Meeting, Paris, France  
United Biomedical Inc., Hauppauge, New York



**Other Activities:**

- H. Caldwell - NIAID Study Section, Bacteriology and Mycology, Ad hoc member  
National Advisory Allergy and Infectious Diseases Council  
NIAID Promotion and Tenure Committee  
NIAID Vaccine Advisory Committee
- T. Hackstadt - NIAID Study Section, Bacteriology and Mycology, Ad hoc member  
National Science Foundation, Ad hoc reviewer  
NIAID, RML, Biosafety Committee, Chair and Institutional Biosafety Officer  
NIAID, RML, Safety Committee  
NIAID, RML, Library Committee
- R. Morrison - NIAID Study Section, Bacteriology and Mycology, Ad hoc member  
NIAID Study Section, Tropical Medicine and Parasitology, Ad hoc member  
Merit Review Board, Department of Veterans Affairs, Ad hoc member  
Wellcome Trust, London, England, Ad hoc member  
Swiss National Science Foundation, Ad hoc member  
NIAMS Study Section, Arthritis and Musculoskeletal and Skin Diseases, Ad hoc member  
NIAID, RML, Animal Care and Use Committee  
NIAID, RML, Biosafety Committee





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  ZO1 AI 00216-14 LICP
PERIOD COVERED October 1, 1993 to September 30, 1994		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Chlamydial Vaccine Development		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	H.D. Caldwell	Chief LICP, NIAID
Others:	H. Su	Visiting Associate LICP, NIAID
	T. Cotter	IRTA LICP, NIAID
	J. Simmons	Bio. Lab Tech. LICP, NIAID
COOPERATING UNITS (if any)  Dr. Andrew Murdin, Connaught Laboratories, Willowdale, Ontario, Canada		
LAB/BRANCH Laboratory of Intracellular Parasites, Hamilton, Montana 59840		
SECTION Immunology of Intracellular Parasites		
INSTITUTE AND LOCATION NIAID, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.3	2.4	1.9
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided..)		
<p>             The objective of this project is the development of subunit or recombinant vaccine to prevent infections caused by <i>Chlamydia trachomatis</i>. Chlamydiae are a major cause of sexually transmitted diseases (STDs) and trachoma which remains the worlds leading cause of preventable blindness. A vaccine capable of preventing chlamydial infection or reducing chlamydial disease sequelae is badly needed. Chlamydial infections are restricted to the oculogenital mucosae and are caused by multiple chlamydial serovars. Local antibody (slgA) plays an important role in protection against chlamydial colonization and infection of mucosal epithelial cells. The goal of this work is to generate a subunit or recombinant chlamydial vaccine capable of evoking broadly cross-protective anti-chlamydial neutralizing at the oculagenital mucosae. Towards this end we have identified key T-helper and B-cell neutralizing epitopes of the chlamydial major outer membrane protein (MOMP); the principle neutralizing antigen of chlamydiae. Several approaches are being used to target these key MOMP antigenic determinants to evoke a mucosal immune response. These include incorporation of the epitopes as chemical and gene fusions with the B subunit of cholera toxin and the construction of recombinant polioviruses expressing MOMP epitopes as gene fusions with the poliovirus major capsid protein VP1. Our most promising results have been with recombinant poliovirus vectors. Recombinant poliovirus expressing antigenically common MOMP neutralizing epitopes grew nearly as well as parent virus and were highly immunogenic in rabbits evoking high titered serum anti-chlamydial antibodies that were broadly cross neutralizing <i>in vitro</i>. Future work will involve evaluating the protective efficacy of the poliovirus/MOMP chimeric virus in a non-human primate animal model of <i>C. trachomatis</i> ocular and genital tract infection.           </p>		



## Background and Objectives:

Oculogenital infections caused by *C. trachomatis* are a leading cause of sexually transmitted diseases and preventable blindness. A safe and effective vaccine is needed to prevent or control chlamydial diseases. The major outer membrane protein (MOMP) is the most promising antigen for the development of chlamydial vaccine. Our approach towards the development of a chlamydial vaccine is to (i) identify key antigenic determinants of the MOMP that are capable of eliciting broadly cross-reactive neutralizing antibodies, (ii) construct inert, toxin based, and infectious viral vectors that are capable of targeting mucosal immunity against these MOMP antigenic determinants, and (iii) to evaluate the protective efficacy of promising vaccine candidates in a non-human primate model of *C. trachomatis* mucosal infection.

## Major Findings:

The MOMP genes of *C. trachomatis* serovars are characterized by four symmetrically spaced variable domains (VDI-IV) that are flanked by five sequence conserved segments (CSI-V). The MOMP VDs are surface accessible and sequences contained within the VDs are targets of chlamydial neutralizing antibodies. In past work, we have identified at the molecular level a highly conserved neutralizing septameric epitope (LNPTIAG) that resides within the central region of MOMP VDIV. This epitope is an attractive candidate for the development of chlamydial vaccine since it is common to most *C. trachomatis* serovars. We have shown that parenteral immunization of mice with a chimeric peptide containing a conserved T-helper cell epitope of the MOMP and the species common LNPTIAG neutralizing epitope is capable of evoking high titered anti-chlamydial neutralizing antibodies *in vitro*. Furthermore, the chimeric peptide was shown to be immunogenic in many congenic mouse strains differing at H-2, and to function as a potent priming immunogen for the re-call of high titered neutralizing antibodies following a secondary re-challenge with intact chlamydiae. In this past year, we have completed studies evaluating the protective efficacy of parenterally administered peptide in both murine and non-human primate models of chlamydial genital and ocular infections. Our data show that humoral (serum) neutralizing IgG antibodies generated against the synthetic peptide antigen were only moderately protective against chlamydial genital tract or ocular challenge. Vaccinated animals were partially protected against low chlamydial challenge inocula as shown by both reduced levels of infection (colonization) and shedding. Protection correlated with low levels of chlamydial specific IgG in secretions which probably localized to the mucosae by transudation of serum antibodies. However, the protective effect of local IgG was overwhelmed by increasing the chlamydial challenge dosage. When higher challenge inocula were administered there was no effect on either chlamydial colonization or shedding. The rather poor efficacious nature of humoral neutralizing IgG antibodies is not particularly a surprising or unexpected finding since chlamydial infection is localized to mucosal surfaces. If fact, the data are encouraging since they show that local antibody can be effective in preventing chlamydial mucosal colonization which has not been demonstrated previously in other chlamydial vaccine studies. Furthermore, they indicate that if the vaccine can be targeted to evoke chlamydial specific mucosal immunity (sIgA) it is possible that greater levels of protection can be achieved.

A number of systems have been investigated to achieve active mucosal immunization against the chlamydial peptide vaccine or key B-cell epitopes contained in its sequence. These include: (i) microencapsulation of chimeric T:B cell peptides into the biodegradable copolymer poly-DL-lactide-co-glycolide, (ii) chemical conjugation of the peptide to CTB and the construction peptide CTB gene fusions, and (iii) expression of MOMP neutralizing epitopes as poliovirus VP1 chimeras. To date, the most promising of these is recombinant poliovirus expressing MOMP neutralizing epitopes. Poliovirus chimeras expressing MOMP neutralizing epitopes in the poliovirus VP1 NAg1 neutralizing site have been generated and characterized in terms of their growth characteristics, antigenic properties, and



immunogenicity. The results are extremely encouraging. Poliovirus/MOMP hybrids possess growth properties that very similar to wild type virus, are neutralizable by chlamydial specific antibodies, and most notably, immunization with the hybrid virus produced markedly higher and more consistent neutralizing antibody titers than those obtained with equimolar amounts of chimeric peptide, purified MOMP, or intact chlamydial organisms.

### **Proposed Course of Work:**

In the upcoming year we will continue to focus our efforts on designing methods to deliver MOMP chimeric T:B cell peptides or neutralizing MOMP epitopes to elicit mucosal immunity, and evaluating the protective efficacy of promising immunogens in a sub-human primate model of chlamydial infection. We will continue to investigate the use of microencapsulated peptides since this technology has been shown by other investigators to be a useful system for mucosal immunization. Future work will involve peroral vaccination of mice with copolymer microencapsulated peptides to determine if this approach will provide an efficient mucosal immunization strategy. Concurrently, we will also continue to work on the construction of recombinant CTB expressing MOMP neutralizing epitopes. Our preliminary work has shown that CTB subunit MOMP peptide fusions fail to assemble and thus do not form soluble B-subunit pentamers which are required for effective stimulation of mucosal immunity. In an effort to obtain pentamerization of recombinant CTB expressing MOMP epitopes a novel strategy was developed in which fusions were made with the CT A subunits. Preliminary data indicate that expression of this genetic construction results in the production of holotoxin in which the MOMP epitope is fused to the A subunit, which associates with wild type pentameric B-subunits. Further biochemical analyses will be conducted to confirm that these novel fusions form functional holotoxins. The ability of microencapsulated peptide and recombinant LT expressing MOMP sequences to evoke mucosal neutralizing antibodies will be initially tested in the mouse. Immunogens will be administered perorally and immunogenicity quantified by sIgA anti-chlamydial specific ELISPOT assays using lymphocytes isolated from the Peyer's patches, mesenteric nodes, and lamina propria. If one or more of these approaches are shown to be effective in eliciting mucosal anti-chlamydial neutralizing antibodies they will then be used to vaccinate sub-human primates to assess immunogenicity and protective efficacy.

A major effort will be placed on evaluating recombinant poliovirus/MOMP hybrids as live vaccine vectors for mucosal immunization since to date this seems to be the most promising approach. Poliovirus/MOMP chimeras are particularly attractive because attenuated vaccine strains of poliovirus have a long history of safe usage in humans, and infection with the virus induces a strong and sustained mucosal immune response. Additionally, cynomolgus monkeys are naturally susceptible to poliovirus infection and are also a well established model for chlamydial infection. As described above we have produced recombinant poliovirus/MOMP hybrids using the virulent poliovirus type 1 (Mahoney) strain. The recombinant virus grows nearly as well as wild type virus and is highly immunogenic (e.g., evokes the production of high titered anti-chlamydial neutralizing antibodies). We will now engineer these same MOMP neutralizing epitopes onto the capsid of the poliovirus type 1 Sabin vaccine strain, using the protocols developed for the construction wild type poliovirus/MOMP recombinants. The recombinant vaccine poliovirus hybrids will be similarly characterized as to their *in vitro* growth properties and immunogenicity. The availability of vaccine-strain polio/MOMP hybrids will make it possible to infect monkeys, characterize the mucosal and humoral immune response to the MOMP epitopes, and then directly evaluate protective efficacy by ocular chlamydial challenge.





**Publications:**

Su, H., and Caldwell, H.D.: Immunogenicity of a Synthetic Oligopeptide Corresponding to Antigenically Common T-Helper and B-Cell Neutralizing Epitopes of the Major Outer Membrane Protein of *Chlamydia trachomatis*. Vaccine 11: 1159-1166, 1993.

Su, H., and Caldwell, H.D.: Immunogenicity of a Synthetic Oligopeptide Corresponding to Species Common T-helper and B-cell Neutralizing Epitopes of the MOMP of *Chlamydia trachomatis*. R.M. Chanock, R. A. Lerner, F. Brown and H. Ginsberg (Eds.), Vaccines 93, Modern Approaches to New Vaccines. Cold Spring Harbor Laboratory, 1993.

Murdin, A.D., Su, H., Stewart, S., Klein, M.H., and Caldwell, H.D.: A Poliovirus Hybrid Expressing a Neutralization Epitope from the Major Outer Membrane Protein of *Chlamydia trachomatis* Is Highly Immunogenic. Infect. Immun. 61: 4406-4414, 1993.

**Patents:**

Caldwell HD, Hitchcock PJ. US Patent 6-696,638: Monoclonal antibodies against chlamydial genus specific lipopolysaccharide, pending.

Nano FE, Caldwell HD. US Patent 6-707,012: Recombinant clones of *Chlamydia trachomatis* lipopolysaccharide, pending.

Caldwell HD, Ying Y, Zhang Y-X, Watkins NG. US Patent 7-324-664: Nucleotide and deduced amino acid sequences of the four variable domains of the 15 serovars of *Chlamydia trachomatis*, pending.

Morrison RP and Caldwell HD. US Patent 5,071,962: Nucleotide and deduced amino acid sequence, isolation and purification of heat-shock chlamydial proteins, pending.

Caldwell HD and Su H. US Patent 7-853,359: Nucleotide and Amino Acid Sequences of the Four Variable Domains of the Major Outer Membrane Proteins of *C. trachomatis*, pending.

Caldwell HD and Su H. US Patent 7-947,671: Synthetic peptide vaccine for *Chlamydia trachomatis*, pending.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 AI 00519-07- LICP

## PERIOD COVERED

October 1, 1993 to September 30, 1994

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunopathogenesis of Chlamydial Infections

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R.P. Morrison Microbiologist LICP, NIAID

Others: K. Lyng Chemist LICP, NIAID

## COOPERATING UNITS (if any)

Univ. Toronto, Ontario, Canada (R. Innam); Univ. Wisconsin Med. Sch., Madison, WI (G.Byrne, W.Beatty); New York Hosp.-Cornell Med. Ctr., New York, NY (S. Witkin); University of Montana, Division of Biological Science, Missoula, Montana (Y. Ying). Indiana Univ. School of Medicine, Indianapolis, Indiana (R.Jones, J.Arno).

## LAB/BRANCH

Laboratory of Intracellular Parasites, Hamilton, Montana 59840

## SECTION

Immunology of Intracellular Parasites

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

1

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

*Chlamydia trachomatis* is a major cause genital tract infections, and in women, these infections often lead to serious complications such as, salpingitis, tubal blockage and infertility. Although immunity to chlamydial infection has been studied for over 20 years, our understanding of the precise role of the immune responses elicited following infection are inadequately defined. Gene targeting has been used to produce mice with disrupted  $\beta_2$  microglobulin ( $\beta_2M^{-/-}$ ), I-A (class II $^{-/-}$ ), or CD4 (CD4 $^{-/-}$ ) genes. These mice are devoid of cell surface expression of MHC class I, MHC class II and CD4 molecules, respectively.  $\beta_2M^{-/-}$  mice are deficient in CD8 $^{+}$  cytotoxic T lymphocytes, class II $^{-/-}$  mice lack helper T cells (CD4 $^{+}$ ), and CD4 $^{-/-}$  mice have reduced levels of helper T cell activity. In this study we examined the capacity of those immunodeficient mouse strains to resolve *Chlamydia trachomatis* genital tract infection. C57BL/6 and  $\beta_2M^{-/-}$  mice resolved their infections similarly, and were culture negative by 4 to 5 weeks following infection. However, class II $^{-/-}$  mice failed to resolve infection and CD4 $^{-/-}$  mice showed a delay (2 weeks) in the resolution of chlamydial genital tract infection. Secondary challenge of  $\beta_2M^{-/-}$  and CD4 $^{-/-}$  mice established that acquired immunity developed in these mice, that was characterized by an infection of shortened duration and reduced shedding of infectious chlamydiae. Serological analysis of C57BL/6 and  $\beta_2M^{-/-}$  mice by ELISA revealed no striking differences in anti-EB IgM, IgG1, IgG2a, IgG2b, IgG3 or IgA antibodies, although some differences were observed in the magnitude of the IgG2a and IgG2b responses. Conversely, class II $^{-/-}$  mice produced far less anti-chlamydial antibodies of all isotypes, and CD4 $^{-/-}$  mice had antibody responses similar to C57BL/6 mice except the IgA response in CD4 $^{-/-}$  animals was delayed and of lower titer. Analysis of vaginal washes for anti-chlamydial antibodies revealed the presence of IgG2a, IgG2b and IgA in C57BL/6 and  $\beta_2M^{-/-}$  mice, no anti-chlamydial antibodies in class II $^{-/-}$  mice, and primarily IgA in CD4 $^{-/-}$  mice. Furthermore, the appearance of chlamydial-specific IgA in the vaginal wash of CD4 $^{-/-}$  was delayed and its appearance coincided with decreased chlamydial shedding and resolution of infection. C57BL/6 and class II $^{-/-}$  mice were tested for chlamydial-specific delayed hypersensitivity responses. Chlamydial specific delayed hypersensitivity was not demonstrated in class II $^{-/-}$  mice. These results clearly demonstrated that MHC class I restricted CD8 $^{+}$  cytotoxic T cells were not required for the resolution of chlamydial genital tract infection. Furthermore, cell surface expression of MHC class II molecules was found to be essential for the development of protective immunity to chlamydial infection, and a strong correlation exists between chlamydial specific IgA in vaginal secretions and recovery from genital tract infection.



## Background and Objectives:

Chlamydial infections constitute significant public health problems worldwide. Trachoma, an ocular infection caused by *Chlamydia trachomatis*, is a major cause of blindness in developing countries. Chlamydial genital tract infections are common worldwide, and in women, these infections often lead to serious complications such as, salpingitis, tubal blockage and infertility. Although immunity to chlamydial infection has been studied for over 20 years, our understanding of the precise role of the immune responses elicited following infection are inadequately defined.

Studies of *C. trachomatis* infection in humans and experimental animals provide evidence that protective immunity develops following infection. In experimental animals, protection is evident for several months following infection, but resistance wanes and reinfection is possible. Protective immunity to human chlamydial genital tract infection is less well documented. However, data from some studies support the notion that protective immunity does develop, and is of limited duration. Both humoral and cell-mediated immune (CMI) responses are activated following chlamydial infection. Anti-chlamydial antibodies are found in serum and secretions, and secretory antibody is thought to play a primary role in protection. The development of cell-mediated immune responses, as defined by chlamydial-specific T cell proliferation or delayed hypersensitivity responses, also correlates with the resolution of infection.

Various animal models have been used to evaluate the contribution of CMI to the resolution of chlamydial genital tract infection. The literature is replete with conflicting observations, and a consensus has not been achieved regarding the functional role of T cells in chlamydial immunity. For example, it has been reported that genital infection of T cell deficient nude mice with *C. trachomatis* results in a chronic infection that persists for > 9 months, and that adoptive transfer of spleen cells enriched for either T or B cells brings about the resolution of infection. In contrast to those studies others have reported that nude mice resolve genital chlamydial infection at the same time as immunologically intact animals, and that adoptive transfer of immune cells does not confer protection. It must be pointed out however, that experimental design and the infecting strain of *C. trachomatis* differs from laboratory to laboratory, and may account for the observed differences. Nevertheless, a consensus has not been reached regarding the role of humoral and cellular immune responses in the resolution of chlamydial infection.

Studies to address the relative contribution of T cell subpopulations in resolving chlamydial infection have also been inconclusive. In vivo depletion of CD4<sup>+</sup> or CD8<sup>+</sup> T cells with mAbs, or the passive transfer of T cell lines/clones have resulted in marginal effects on the resolution of genital tract infection. An alternative approach to identifying cellular immune responses that function in immunity to infectious diseases is to use gene knockout mice. Gene targeting is a method by which specific genes are altered in embryonic stem cells and subsequently passed through the germ line. This method has been used to produce mice that are devoid of cell surface expression of MHC class I, class II and CD4 molecules. MHC class I deficient animals have been generated by inactivation of the gene for  $\beta_2$ -microglobulin ( $\beta_2$ M), which is required for the proper assembly and cell surface expression of the MHC class I molecule, and as a result these mice are deficient in CD8<sup>+</sup> T cells. Mice that are devoid of cell surface expression of MHC class II molecules (class II<sup>-/-</sup>) are prepared by inactivation of the I-A<sub>b</sub> gene, which results in mice that are deficient in CD4<sup>+</sup> helper T cells. Targeted disruption of the CD4 gene results in mice that have impaired development of the CD4 T cell lineage and decreased helper T cell activity.

The primary objective of our studies is to determine which lymphocyte sub-population(s) plays a predominant role in the resolution of chlamydial genital tract infection. Toward this goal, we have examined the capacity of  $\beta_2$ M<sup>-/-</sup>, class II<sup>-/-</sup> or CD4<sup>-/-</sup> mice to resolve *C. trachomatis* genital tract infection, and have assessed both humoral and cell-mediated aspects of the anti-chlamydial immune response.

## Major Findings:

The ability of normal and gene knockout mice to resolve chlamydial genital tract infection was assessed by isolation of infectious chlamydiae at numerous times following vaginal inoculation. C57BL/6 and  $\beta_2$ M<sup>-/-</sup> mice resolved chlamydial genital tract infection similarly, and all mice were culture negative 4-5 weeks post infection. Conversely, class II<sup>-/-</sup> mice remained culture positive throughout the study (>70 days), and no significant decrease was observed in the shedding of infectious chlamydiae. CD4<sup>-/-</sup> mice recovered from primary infection, but the resolution of infection was significantly delayed (2 weeks). C57BL/6,  $\beta_2$ M<sup>-/-</sup> and CD4<sup>-/-</sup> mice that recovered from a



**Proposed Course of Work:**

Dr. Morrison is leaving the RML 7/94 and therefore this project will be terminated.

**Publications:**

Beatty, W. L., Byrne, G. I. and Morrison, R. P.: Repeated and persistent infection with *Chlamydia* and the development of chronic inflammation and disease. Trends in Microbiology 1994; 2:94-98.

Beatty, W.L., Belanger, T.A., Desai, A.A., Morrison, R.P., and Byrne, G.I.: Role of tryptophan in  $\gamma$ -interferon mediated chlamydial persistence. New York Acad. Sci. in press.

Beatty, W.L., Belinger, T.A., Desai, A.A., Morrison, R.P., and Byrne, G.I.: Tryptophan depletion as a mechanism of gamma interferon mediated chlamydial persistence. Infection and Immunity, in press

Beatty, W.L., Morrison, R.P., and Byrne, G.I.: Immunoelectron microscopic quantitation of differential levels of chlamydial proteins in a cell culture model for persistent *Chlamydia trachomatis* infection. Infection and Immunity, in press.

**Patents:**

Morrison RP and Caldwell HD. US Patent 5,071,962: Nucleotide and deduced amino acid sequence, isolation and purification of heat-shock chlamydial proteins. Date issued December 10, 1991.





<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b>		<b>PROJECT NUMBER</b>	
<b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>ZO1 AI 00552-06 LICP</b>	
<b>PERIOD COVERED</b>			
October 1, 1993 to September 30, 1994			
<b>TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)</b>			
Immunopathobiology of bacterial toxins			
<b>PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)</b>			
<b>PI:</b>	W. Cieplak, Jr.	<b>Unit Head</b>	LICP, NIAID
<b>Others:</b>	C.R. Grant	<b>Visiting Associate</b>	LICP, NIAID
	R. Messer	<b>Microbiologist</b>	LICP, NIAID
<b>COOPERATING UNITS (if any)</b>			
Boyd E. Haley (University of Kentucky); Richard Kahn (NCI, NIH); Rockford K. Draper (University of			
<b>LAB/BRANCH</b>			
Laboratory of Intracellular Parasites, Hamilton, Montana 59840			
<b>SECTION</b>			
Molecular Pathogenesis Unit			
<b>INSTITUTE AND LOCATION</b>			
NIAID, NIH, Bethesda, MD 20892			
<b>TOTAL MAN-YEARS:</b>	<b>PROFESSIONAL:</b>	<b>OTHER:</b>	
2	1	1	
<b>CHECK APPROPRIATE BOX(ES)</b>			
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews			
<b>SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)</b>			
<p>Analyses of the mutant analogs of Escherichia coli heat-labile enterotoxin, a close relative of cholera toxin, have revealed that alterations which abrogate ADP-ribosyltransferase activity also impart conformational changes that are detectable by tryptic fragmentation patterns. However, conservative substitution at tryptophan 170 by tyrosine yields a molecule with reduced activity and apparent wild-type conformation as judged by trypsin sensitivity. This mutation, in conjunction with other previously described substitutions may be a suitable target for the generation of, multiply substituted mutant proteins that are devoid of toxic/enzymatic activity and that retain native conformation. We are investigating various amino acid substitutions at various targets in the hope of generating mutants with the appropriate phenotypic characteristics (i.e. no enzymatic activity and no gross conformational alterations).</p> <p>Additional studies have been conducted to evaluate the effect of post-translational modifications on the enzymatic activity of the A subunit of LT. These studies have revealed that limited proteolytic cleavage is not absolutely necessary for the expression of enzymatic activity and that multiple auto-or self ADP-ribosylation does not detectably change the specific enzymatic activity of the toxin. These findings contrast the results of studies conducted by other groups using cholera toxin. Further studies have investigated the manner in which LT is trafficked in eukaryotic cells using mutant analogs and mutant cell lines. These studies have revealed that the toxin likely does not enter cells using a Golgi-dependent retrograde pathway and probably gains access to the target substrates (G proteins) via an early endosomal compartment.</p>			





## Background and Objectives:

The principal goal of these studies is to identify the primary structural requirements attendant to the enzymatic and toxic activities of members of the cholera toxin family of enterotoxins. This family includes toxins from both *Vibrio cholera* and the closely related heat-labile enterotoxins (LT) from *Escherichia coli*. The family may also include other less well described toxins from other enteric pathogens. The toxins exert their biological effects on eukaryotic cells through the NAD-dependent ADP-ribosylation of GTP-binding regulatory proteins of the adenylate cyclase complex, and are thought to be the primary mediators in the pathogenesis of diarrheal disease caused by *V. cholera* and various enterotoxigenic *E. coli* strains. Accordingly, the toxins are considered to be important antigens for inclusion in potential acellular vaccines for such diseases. In addition, these toxins possess the ability to potentiate intestinal mucosal immune responses to unrelated antigens when given orally. The relationship between enzymatic/toxic activity and the immunopotentiating properties has not been clearly established. Our studies are, therefore, designed to identify residues or regions of LT that are important to enzymatic and toxic activity by using site-directed mutagenesis and other biochemical approaches. The resultant information will then be used to create stable, genetically detoxified mutant analogs that may be suitable for vaccine use. Further, such analogs will permit the precise evaluation of the role of enzymatic activity in the immunomodulatory activities of LT and related toxins.

## Major Findings:

### Active site of LT

The recent publication of the X-ray crystal structure of LT, refined to 1.95 Å resolution, has allowed us to investigate the potential involvement of various residues in a predicted NAD-binding cleft. The refined crystal structure has also shown that arginine 7, a residue we previously showed to be involved in enzymatic activity, lies within the predicted NAD-binding site. More recently, we have altered histidine 44, serine 61, alanine 69, tryptophan 127, and a number of other residues using multiple isoionic or isosteric substitutions; these residues are predicted to lie within or to be vicinal to the substructure or cleft presumed to be the NAD<sup>+</sup>-binding site or to be associated with catalytic subsites. While many substitutions at these residues are capable dramatically reducing enzymatic activity, retention of gross conformation, as judged by sensitivity to limited trypsinolysis, is dependent upon the type of substitution introduced. Further analyses of effects on conformation and substrate binding are awaiting the installation of a microcalorimeter capable of isothermal and differential scanning measurements. Mutations that have been judged to result in reductions of enzymatic activity but no detectable alterations in overall conformations will be introduced into the gene for the holotoxin to assess their effects on toxin assembly and cytotoxic effects.

We have begun analyses of potential residues involved in the NAD<sup>+</sup>-binding site using photolabeling techniques. These studies have been facilitated by the development of an FPLC purification scheme that results in preparations of recombinant A subunits and analogs of greater than 95% purity. Direct photolabeling with [carbonyl-<sup>14</sup>C]-NAD and ultraviolet irradiation has been used and occurs at a level of 20 - 30 mol %. The labeling is saturable, time dependent, and results in the specific inactivation of enzyme activity. Labeled peptides are being isolated and will be subjected to microsequence analysis to determine the actual site of photolabeling; preliminary analyses using proteolytic fragmentation of the labeled A subunit indicates that the vast majority of the label lies in a peptide containing two glutamic acid residues (110 and 112) that are thought to be critical to catalytic activity. In a collaborative effort with Dr. Boyd Haley (University of Kentucky), we are also employing photoactivatable derivatives of NAD (2-azido-NAD) to map the NAD<sup>+</sup>-binding site. To date, effective photolabeling of recombinant LT A subunit has been achieved at low temperature. Interestingly, both direct photolabeling and photolabeling using 2-azido-NAD have revealed that a co-factor, termed ADP-ribosylation factor (ARF), that is required for the activity of LT and CT appears to bind to the toxins at or near the NAD<sup>+</sup>-binding site.



### Effects of Post-translational modification of LT-A subunit on enzymatic activity

As noted in the previous report, studies in our laboratory using isolated recombinant A subunit have shown that proteolytic cleavage of the A subunit of LT, long presumed to be requisite to enzymatic activity and toxicity, is not absolutely required for either activity. However, more detailed analyses of the induction of cAMP increases in cells shows that blocking tryptic cleavage by substitution of a critical arginine residue results in a significant increase in the lag time required for activation of adenylate cyclase. This result suggests that intra- or peri-cellular tryptic activation may play a role in the intoxication and may explain the differences in severity associated with infections associated with enterotoxigenic *E. coli* and those of *V. cholerae*. In addition, we have observed that under conditions of high level expression in *E. coli*, the A subunit undergoes extensive auto or self-ADP-ribosylation at various arginine residues, as judged by alterations in electrophoretic mobility and the sensitivity of these alterations to treatment with neutral hydroxylamine. Previous observations of cholera toxin, auto-ADP-ribosylated in vitro, have suggested that such modification is accompanied by increases in specific enzyme activity. Interestingly, our analyses of the different ADP-ribosylated forms of the A subunit of LT isolated by FPLC indicates that this modification has little or no influence on the activity of the molecule.

### Role of the endoplasmic reticulum and Golgi complex in toxin internalization

Recent work from our laboratory and elsewhere has indicated that both cholera toxin (CT) and LT exert their toxic effects through a pathway that is sensitive to brefeldin A, a fungal metabolite that disrupts Golgi function. The A subunits of both CT and LT possess a carboxyl-terminal tetrapeptide sequence (KDEL in CT, RDEL in LT) that is known to function as an endoplasmic reticulum retention sequence in eukaryotic cells. These observations have led to the proposal that these and related toxins gain access to G protein targets via a pathway that involves retrograde transport through the Golgi-endoplasmic reticulum complex. We have introduced mutagenic changes in the RDEL sequence of the LT-A subunit that are expected to disrupt the retention function of the sequence and have found that such alterations have only a nominal effect on toxicity. Further, we have examined the ability of LT and CT to intoxicate a mutant CHO cell line, designated V.24.1, that possesses a temperature-sensitive lesion in Golgi structure and function. This cell line responds to LT and CT in a fashion equivalent to that of wild-type cells at the restrictive temperature. Collectively, these results indicate little or no role for the main Golgi stacks and endoplasmic reticulum in the intracellular trafficking of these toxins and point to a role for an early endosomal or trans-Golgi compartment in intoxication.

### **Proposed Course of Work:**

The analyses of a considerable number of individual mutagenic substitutions on the enzymatic and conformational properties of recombinant A subunit analogs is nearing completion. Suitable combinations of the mutants (double substitutions) will be employed to render holotoxins devoid of toxic properties. Such holotoxins will be evaluated for both in vitro and in vivo toxic effects using cell culture models and animals. Collaborations with laboratories well-versed in the analyses of mucosal immune responses are being planned with emphasis on examining the efficacy of detoxified mutant analogs on mucosal adjuvant activity, particularly with respect to the elicitation of mucosal immune responses to Chlamydial epitopes.

Additional studies will continue to focus on identifying regions or sequences in the A and B subunits involved in the internalization of the toxin by eukaryotic cells. It can be hypothesized that translocation of the A subunit to the cytosol may use a pathway involving endosomal vesicles and/or the trans-Golgi complex. Wild-type and mutant toxins (i.e. RDEL mutants) will be labeled with fluorescent tracers and intracellular locations of the toxins will be monitored by laser confocal microscopy.

### **Publications:**

None



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  ZO1 AI 00553-05 LICP
PERIOD COVERED October 1, 1993 to September 30, 1994		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Biology and Immunology of Pathogenic <i>Campylobacter</i> spp.		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)		
PI:	W. Cieplak, Jr.	Unit Head LICP, NIAID
Others:	M.E. Konkel D.J. Mead	IRTA Microbiologist LICP, NIAID LICP, NIAID
COOPERATING UNITS (if any)  Stanford University Medical School (Lucy Tompkins); University of Arizona (Lynn Joens).		
LAB/BRANCH Laboratory of Intracellular Parasites, Hamilton, Montana 59840		
SECTION Molecular Pathogenesis Unit		
INSTITUTE AND LOCATION NIAID, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1	1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             We have focused our attention on the interaction of <i>Campylobacter jejuni</i> with human epithelial cells particularly with respect to altered protein synthesis that occurs during the binding and internalization of <i>C. jejuni</i>. Antisera prepared against organisms grown in the presence of epithelial cells are capable of inhibiting translocation and have been used to screen recombinant expression libraries of <i>C. jejuni</i> genomic DNA. Several clones have been identified that produce proteins that are preferentially recognized by antisera produced against bacteria cultured with epithelial cells. One clone was completely sequenced and appears to encode the homolog of the low molecular weight, bacterial DNA-binding protein HU. The promoter structure and other regulatory elements of the gene have been mapped. In addition, we have identified and characterized an intron-like element in the 23S ribosomal subunit genes of <i>C. jejuni</i>. Additional studies have characterized in vitro phenotypic passage variants of <i>C. jejuni</i>. A high passage variant of a clinical isolate termed M96 that is defective for internalization within epithelial cells has also been obtained. However, this variant is fully competent at translocating across monolayers of polarized epithelial cells. At the molecular level the strain differs only from the parent in terms of the structure of lipopolysaccharide (LPS). The specific differences in structure are being characterized.           </p>		





## Background and Objectives:

The principal goal of these studies is to define in molecular terms the pathogenic mechanisms associated with enteritis caused by *Campylobacter jejuni*. *C. jejuni* is now recognized as one the most common causes of human enteritis/diarrhea in both developed and developing nations. In addition, antecedent *C. jejuni* infection is now known to be frequently associated with Guillain-Barré polyneuropathy. Despite this increased recognition of *C. jejuni* as a significant human pathogen, little is known about the pathogenic mechanisms and virulence properties associated with this organism. Elucidation of the virulence properties of this organism will also yield insights into the more general phenomenon of bacterial pathogenesis, particularly with respect to enteric disease, a major cause of morbidity and mortality in the world.

## Major Findings:

Previous studies have shown that *C. jejuni* alters its protein synthetic profile during interaction with epithelial cells in culture. These observations prompted us to investigate the nature of the proteins that are novel synthesized or enhance during the processes of binding and internalization. To facilitate characterization of the genes we have created several expression libraries of *C. jejuni* genomic DNA. We have been able maintain genomic fragments in a stable fashion through use of a methyl-mismatch repair deficient strain of *E. coli* as the host. Genomic clones have been screened for preferential reactivity using antisera generated against bacteria grown in the presence and absence of epithelial cells. To date, several clones reacting preferentially with antisera prepared against bacteria cultivated with epithelial cells have been identified. One clone and derivatives have been completely sequenced and they appear to encode the homolog of the widespread bacterial DNA-binding protein termed HU. The preferential immunoreactivity of this protein is somewhat puzzling since it is normally quite abundant during bacterial growth. It is noteworthy, however, that the HU gene is only the fifth *C. jejuni* gene to be cloned and sequenced, and as such provides additional information regarding the characteristics of promoters and other regulatory elements of genes from this organism.

Several additional clones have been identified that encode proteins that react with one or both antisera referred to above. One clone produces another non-HU low molecular weight protein (c.a. 20,000) that may represent an immunodominant antigenic determinant on the surface of the organism. The sequence of this gene is nearly complete. Additionally, we have identified an intervening sequence in the 23S ribosomal RNA genes of *C. jejuni*. This sequence is unique in that it forms a rather complex secondary structure reminiscent of other mobile genetic elements is excised from the RNA after transcription.

Prolonged *in vitro* passage of a *C. jejuni* isolated has been used to generate a phenotypic passage variant that is significantly impaired in the ability to enter conventionally cultured human epithelial cells. However, this variant is fully capable of translocating across barriers formed by monolayers of polarized epithelial cells (Caco-2). The passage variant differs from the parent strain in the molecular weight of its lipopolysaccharide (LPS) and genomic restriction enzyme profile. However, no obvious differences in surface, or total protein profile are apparent. These findings indicate that LPS may play an important role in the ability of *C. jejuni* to enter epithelial cells and suggests that LPS, as in the case of other pathogenic bacteria, is an important virulence determinant.

## Proposed Course of Work:

To complement our approach of molecular cloning of selectively expressed genes and characterization of passage variants, we are in the process of developing genetic systems for the creation of stable isogenic mutants of *C. jejuni* that are defective in binding to, internalization within, and





translocation across epithelial cells. This approach involves the construction of mini-transposon vectors (e.g. mini-Tn5 and derivatives) that possess suitable antibiotic resistance markers (e.g. kanamycin resistance from a *Campylobacter* plasmid). In addition, we suspect that available transposase promoters may not be functional in *Campylobacter* and, as such, alternative, regulated promoters are being introduced into such vectors.

Additional efforts will involve the construction of a suitable transcriptional fusion vector systems that will allow direct identification of genes that are preferentially expressed during the interaction of *C. jejuni* with human epithelial cells. This approach will involve the use of resistance genes to antibiotics (e.g. chloramphenicol) that are capable of entering eukaryotic cells. When fused to genes encoding a phenotypic marker (e.g. B-galactosidase) such selectable markers can be used to rescue and identify genes in random fragments of *C. jejuni* DNA that are preferentially transcribed during intra- and pericellular residence.

#### **Publications:**

Konkel ME, Mead DJ, Cieplak, W Jr. Kinetic and antigenic characterization of altered protein synthesis by *Campylobacter jejuni* during cultivation with human epithelial cells. J Infect Dis 168: 948-954, 1993.

Konkel ME, Marconi, RT, Mead DJ, and Cieplak, W. Jr. Cloning and expression of the hup gene encoding a histone-like protein of *Campylobacter jejuni*. Gene, In Press.

Konkel ME, Marconi RT, Mead DJ, and Cieplak W. Jr. Identification and characterization of an intervening sequence with the 23S ribosomal genes of *Campylobacter jejuni*. Mol Microbiol, In Press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 AI 00567-04 LICP

## PERIOD COVERED

October 1, 1993 to September 30, 1994

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cellular Biology of Host-Parasite Interactions

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	T. Hackstadt	Expert	LICP, NIAID
Others:	R. Garzon	IRTA	LICP, NIAID
	R.A. Heinzen	IRTA	LICP, NIAID
	J.D. Sager	Bio. Lab. Tech.	LICP, NIAID

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Intracellular Parasites, Hamilton, Montana 59840

## SECTION

Immunology of Intracellular Parasites Section

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.8

## PROFESSIONAL:

1.8

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

*Chlamydia trachomatis* is the causative agent of several human diseases including trachoma, the leading cause of infectious blindness worldwide, and is the most common form of sexually transmitted disease in the U.S. and developed countries. Chlamydiae are obligate intracellular bacteria that undergo their life cycles entirely within an intracellular vesicle that is not acidified and does not fuse with lysosomes. The chlamydial inclusion seems to be isolated from established routes of intracellular trafficking. Other than vacuoles containing other chlamydiae, no cellular vesicles are known to fuse with the chlamydial inclusion. The inclusion membrane grows throughout the developmental cycle to contain the multiplying bacteria yet the biosynthetic source of the inclusion membrane is unknown. Although chlamydiae obviously acquire essential nutrients from the host cell, the mechanisms for obtaining these across the inclusion membrane are unknown. We have employed a variety of specific probes for various cellular organelles in conjunction with conventional fluorescence and confocal microscopy in an attempt to identify cellular organelles that may interact with the chlamydial inclusion. Our results indicate a direct involvement of the Golgi apparatus in trafficking of sphingolipids to the chlamydial inclusion and imply a close interaction between the chlamydial inclusion and the Golgi network. Modification of the inclusion membrane by insertion of chlamydial proteins has been demonstrated by a novel experimental approach that detected proteins specific to the infected cells but not on purified chlamydiae. The first of what appear to be multiple parasite-specified polypeptides in the inclusion membrane has been cloned, sequenced, and expressed. Collectively, these data identify a chlamydial protein that is released from intracellular chlamydiae to modify the inclusion membrane. This modification is likely essential for the successful multiplication of the parasite.



## Background and Objectives:

*Chlamydia trachomatis* is the causative agent of several human diseases including trachoma, the leading cause of infectious blindness, and is the most common form of sexually transmitted disease in the U.S. and developed countries. Chlamydiae are obligate intracellular bacteria that undergo their life cycles entirely within an intracellular vesicle that is not acidified and does not fuse with lysosomes. There are many fundamental questions regarding the nature of the chlamydial inclusion membrane including its composition, permeability properties, biosynthetic origin and luminal contents. A lack of basic information concerning the physical and nutritional parameters within the chlamydial inclusion severely limits attempts to identify environmental conditions that may serve to regulate the chlamydial developmental cycle. The chlamydial inclusion seems to be isolated from established routes of intracellular trafficking. Other than vacuoles containing other chlamydiae, no cellular vesicles are known to fuse with the chlamydial inclusion. Although chlamydiae obviously acquire essential nutrients from the host cell, the mechanisms for obtaining these across the inclusion membrane are unknown.

## Major Findings:

We have employed a variety of specific probes for various cellular organelles in conjunction with conventional fluorescence and confocal microscopy in an attempt to identify cellular organelles that may interact with the chlamydial inclusion. Information as to possible interactions between the chlamydial inclusion and specific cellular organelles may suggest novel approaches to the study of this unique vesicle. Our results indicate a direct involvement of the Golgi apparatus in trafficking of sphingolipids to the chlamydial inclusion and imply a close interaction between the chlamydial inclusion and the Golgi network.

A fluorescent ceramide analog was used to determine any relationship between the Golgi apparatus and the chlamydial inclusion and to follow sphingolipid metabolism in *C. trachomatis* infected cells. Surprisingly, we found not only close association of the Golgi apparatus with the chlamydial inclusion but the eventual presence of a derivative of this fluorescent probe associated with the chlamydiae themselves. The observation of a fluorescent sphingolipid analog in chlamydiae subsequent to its accumulation in the Golgi, and the inhibition of trafficking of the probe to the chlamydial inclusion by Brefeldin A suggest a direct link between the Golgi apparatus and the chlamydial inclusion. This effect is specific to chlamydiae as an intracellular parasite, *C. burnetii*, inhabiting a lysosomal vesicle does not display similar accumulation of fluorescent sphingolipids. To our knowledge, this is the first demonstration of direct trafficking of an exogenously added substrate to the chlamydial inclusion.

Modification of the inclusion membrane by *C. psittaci* specified proteins was demonstrated through the use of a novel experimental approach that incorporated screening with sera from infected animals after adsorption with elementary bodies (EBs). This effort identified proteins present in infected cells, but absent from the infectious EBs. Immunoblots of lysates of infected HeLa cells probed with the convalescent sera identified protein antigens of 22, 34, and 52 kDa, that were not detected in lysates of purified EB or in uninfected HeLa cells. Immunofluorescent staining of GPIC-infected HeLa cells demonstrated that these adsorbed sera labeled the inclusion and inclusion membrane with no apparent reactivity toward EBs or RBs. To identify these proteins which are unique to the intracellular phase of the chlamydial life cycle, an expression library of *C. psittaci*, GPIC, DNA was screened with antisera from animals which had recovered from infection with GPIC. Overlapping clones expressed a 39 kDa polypeptide (p39). Sequence analysis identified two open reading frames, one of which (ORF 1) coded for a predicted 39 kDa gene product. The ORF 1 coding sequence was fused to the malE gene for expression in *E. coli*. Antisera against the resulting fusion protein were used for immunoblotting and fluorescence microscopy. Immunoblots confirmed that p39 was present in lysates of infected cells and in reticulate bodies (RB), but absent from purified EBs. Additionally, p39 in lysates of infected tissue culture cells had a migration pattern which was suggestive of posttranslational modification. This pattern was not observed in immunoblots of RB or the recombinant *E. coli* clones. Fluorescence microscopy demonstrated that p39 was localized to the inclusion membrane of infected HeLa cells, but the antisera against p39 did not label the developmental forms within the inclusion. In addition to the inclusion membrane, these antisera labeled structures which extended from the inclusion over the nucleus or into the cytoplasm of infected cells. Because the protein produced by ORF 1 is deposited on the inclusion membrane of infected cells, we have called this gene *incA* (inclusion membrane protein A), and its gene product (p39) IncA. Dual-label fluorescence microscopy was conducted with anti-IncA antisera and rhodamine-labeled wheat germ agglutinin, a lectin which binds to the golgi apparatus. These experiments demonstrated that the golgi apparatus is surrounded by the lobed chlamydial inclusion, and that anti-p39 localizes near the golgi within these cells. Collectively, these data identify a chlamydial protein that is released from RB and is localized to the inclusion membrane of infected cells where it may be important for the survival or multiplication of chlamydiae.





Other intracellular parasites, such as *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever, display a very different pathogenic mechanism that involves replication free within the cytoplasm of the host cells. We have recently determined that recruitment and polymerization of host cell actin provides the mechanical force that allows intra- and intercellular spread of spotted fever group rickettsia. This method of cell-to-cell spread is similar to that described for *Shigella flexneri* and *Listeria monocytogenes* and is an elegant mechanism that ensures spread to adjacent cells while avoiding the host humoral immune response.

Rickettsial proteins required for actin polymerization and other virulence determinants are likely synthesized only when the organism encounters a suitable environment. The specific environmental signal(s) and the mechanism(s) of transduction are of great interest. Sensory histidine kinases comprise one component of two-component regulatory systems that sense and transduce diverse environmental stimuli and regulate expression of virulence genes in many pathogenic bacteria. An *R. rickettsii* sensory kinase-encoding gene (*rhkA*) was cloned into *E. coli* using PCR with degenerate oligonucleotides corresponding to conserved regions of known histidine kinases. The *rhkA* reading frame is 1491 bp in length and can direct the synthesis of a 497 aa protein (RhkA) with a predicted Mr of 56,788. Two unique N-terminal His tag fusions were generated to the C-terminus of RhkA allowing purification of fusion proteins by nickel affinity chromatography. Both fusion proteins autophosphorylated in the presence of [ $\gamma$ -<sup>32</sup>P]ATP and the phosphoramidite bond is stable for at least 2 h. Immunoblot analysis using rabbit antiserum against RhkA revealed strong reactivity with RhkA-363 and RhkA-422, and a protein present in *R. rickettsii* lysates. Immunoprecipitation and quantitation of phosphorylated RhkA from purified rickettsia after metabolic activation under different physiological conditions will be conducted to determine what extracellular signal(s) RhkA is responsive to. Investigation of *rhkA* may elucidate important adaptive responses undertaken during the infectious cycle *R. rickettsii*.

We have characterized two outer membrane protein antigens of the spotted fever agent *R. rickettsii*, the *rompA* gene-encoded 224 kDa protein, rOmpA, and the *rompB* gene-encoded 168 kDa protein, rOmpB. Structural features of these proteins suggest possible roles in actin polymerization. The *icsA* protein of *Shigella flexneri* is involved in actin polymerization and, like rickettsial rOmpB, displays cleavage of a C-terminal domain that remains outer membrane associated. Repeat motifs have been associated with bacterial proteins involved in cytoskeletal interactions. The repeat regions of *R. rickettsii* rOmpA will also be given consideration as a possible rickettsial component associating with actin. Transcription and stability of the mRNAs encoding these genes have been described. We will be analyzing temperature dependant expression of these genes since there is some preliminary evidence suggesting that the ratios may be influenced by either growth stage or temperature. Differential gene expression in response to environmental conditions has not been described in rickettsiae. Temperature is an obvious environmental parameter that changes as rickettsia transit from arthropod vectors to vertebrate hosts. If the rOmp A/B ratios shift under different conditions, we will continue to explore mechanisms of transcriptional regulation.

### Proposed Course of Work:

We have begun to address whether the Golgi function of the host cell is affected during chlamydial infection. The protein processing of secreted proteins which transit through the Golgi apparatus is being used as an indicator of Golgi function. Pulse-chase experiments have shown that the rate of processing of the MHC class I polypeptide is slowed in chlamydial infected cells as compared to uninfected control cells. However, this decrease in the rate of processing is not a general phenomena since the rate of processing of VSV G protein and transferrin receptor is unchanged in chlamydial infected cells. Decreased surface expression of MHC class I molecule might provide a mechanism for chlamydial invasion of the host immune system. However, quantitation of MHC class I surface expression has not yet been directly determined.

Immunofluorescence microscopy has demonstrated that during chlamydial infection, transferrin receptor accumulates intracellularly surrounding the chlamydial inclusion. Additionally, exogenously added fluorescently labeled transferrin (Tf) also accumulates and surrounds the inclusion. We are beginning to address how this accumulation occurs? Do chlamydia acquire iron by inhibiting host cell receptor recycling and redirecting Tf(Fe) to the inclusion. Thus, we are determining whether Tf receptor recycling is impaired in chlamydial infected cells.

Finally, experiments are underway to purify intact chlamydial inclusions in order to address certain questions such as 1) composition and origin of chlamydial membrane 2) membrane potential 3) nutrient uptake, 4) requirements for chlamydial replication, etc.





**Publications:**

Hackstadt, T., Scidmore, M., and Rockey, D., Lipid Trafficking in *Chlamydia trachomatis* infected cells: Golgi derived sphingolipids are incorporated into the chlamydial membrane. In: Orfila, J., Byrne, G.I., Chernesky, M.A., Grayston, J.T., Jones, R.B., Ridgway, G., Saikku, P., Schachter, J., Stamm, W.E., and Stephens, R.S. (Eds.): Chlamydial Infections. Cambridge, Cambridge University Press, 1994, pp. 407-410.

Rockey, D.D., Heinzen, R.A., and Hackstadt, T., Fluorescent microscopic examination of Infected Hela cells with antisera directed at an infection-specific chlamydial protein.

In: Orfila, J., Byrne, G.I., Chernesky, M.A., Grayston, J.T., Jones, R.B., Ridgway, G., Saikku, P., Schachter, J., Stamm, W.E., and Stephens, R.S. (Eds.): Chlamydial Infections. Cambridge, Cambridge University Press, 1994, pp. 407-410.

Rockey, D.D., and Rosquist, J.L., Protein antigens of *Chlamydia psittaci* present in infected cells but not detected in the infectious elementary body. Infect. Immun. 62:106-112 (1994).

Policastro, P., and Hackstadt, T., Differential activity of *Rickettsia rickettsii* *ompA* and *ompB* promoter regions in a heterologous reporter gene system. Microbiology (in press)



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b>		<b>PROJECT NUMBER</b> ZO1 AI 00631-03 LICP
<b>PERIOD COVERED</b> October 1, 1993 to September 30, 1994		
<b>TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)</b> Developmental Biology of Intracellular Parasites		
<b>PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)</b>		
PI:	T. Hackstadt	Expert LICP, NIAID
Others:	P.F. Policastro	Senior Staff Fellow LICP, NIAID
	D.D. Rockey	IRTA LICP, NIAID
	R.A. Heinzen	IRTA LICP, NIAID
	M. Scidmore	IRTA LICP, NIAID
	R.E. Mann	Bio Lab Tech. LICP, NIAID
<b>COOPERATING UNITS (if any)</b> None		
<b>LAB/BRANCH</b> Laboratory of Intracellular Parasites, Hamilton, Montana 59840		
<b>SECTION</b> Host-Parasite Interactions Section		
<b>INSTITUTE AND LOCATION</b> NIAID, NIH, Bethesda, MD 20892		
<b>TOTAL MAN-YEARS:</b> 5.0	<b>PROFESSIONAL:</b> 4.0	<b>OTHER:</b> 1.0
<b>CHECK APPROPRIATE BOX(ES)</b> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
<b>SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided..)</b>		
<p>Modification of DNA structure by histone-like proteins appears to be a central regulatory mechanism governing the complex life cycle of <i>Chlamydia trachomatis</i>. These histone homologs, termed Hc1 and Hc2, are expressed only during the late stages of the chlamydial life cycle concomitant with the reorganization of RBs into EBs and play a major role in establishment of nucleoid structure as well as in control of gene expression. Expression of Hc1 in <i>E. coli</i> results in a compaction of the chromatin that ultrastructurally resembles the nucleoid reorganization which occurs late in the chlamydial developmental cycle and produces a global termination of transcription, translation, and replication at concentrations equivalent to that of chlamydial elementary bodies. The association of Hc1 with DNA at levels below that necessary to condense the nucleoid modifies DNA structure/topology to influence promoter activity and gene expression. Detailed biophysical analysis of Hc1-DNA interaction, <i>in vitro</i>, indicate that Hc1 preferentially binds supercoiled DNA. These results imply that Hc1 selectively stabilizes DNA of specific superhelical densities to modify DNA topology. The binding of Hc1 to DNA has been characterized to examine the effects of pH, reducing conditions, and ionic strength on the interaction. Ionic strength had a marked influence on both DNA-binding and DNA-dependent aggregation of the protein. Reversibility of this aggregation was also ionic strength dependent with a discrete optimum ionic strength for maintaining stable association. Hc1 associates with either positively or negatively supercoiled DNA in preference to relaxed DNA implying that the protein has the ability to discriminate between DNA domains of differing topology <i>in vivo</i>. Hc1-mediated effects on DNA topology may thus play a significant role in the regulation of gene expression during chlamydial development. Understanding the controlling mechanisms for gene expression and differentiation may suggest unique methods of interrupting parasite replication.</p>		



## Background and Objectives:

Modification of DNA structure by histone-like proteins appears to be a central regulatory mechanism governing the complex life cycle of *Chlamydia trachomatis*. Histone H1 homologs are rare among prokaryotes, *C. trachomatis* possesses two elementary body specific proteins with primary amino acid sequence homology to eukaryotic H1. These histone homologs, termed Hc1 and Hc2, are expressed only during the late stages of the chlamydial life cycle concomitant with the reorganization of reticulate bodies (RBs) into elementary bodies (EBs) and appear to play a major role in establishment of nucleoid structure as well as in control of gene expression. Alteration of DNA topology in response to environmental signals is thought to play an underlying role in regulation of gene expression in bacteria. The capacity of histone-like proteins to modify chromatin structure and DNA topology is becoming an increasingly recognized as a component of global regulatory schemes involving differential gene expression under various environmental conditions. Hc1-mediated effects on DNA topology may play a significant role in the regulation of gene expression during chlamydial development.

## Major Findings:

Expression of Hc1 in *E. coli* results in a compaction of the chromatin that ultrastructurally resembles the nucleoid reorganization which occurs late in the chlamydial developmental cycle. Hc1 expression in *E. coli* is self-limiting and produces a global termination of transcription, translation, and replication at concentrations equivalent to that of chlamydial elementary bodies. We have proposed that association of Hc1 with DNA at levels below that necessary to condense the nucleoid may exert more specific regulatory effects through modification of DNA structure/topology to influence promoter activity and gene expression. Expression of Hc1 in *C. trachomatis* correlates temporally with a decrease in the superhelical density of the chlamydial plasmid. Expression of Hc1 in *E. coli* also results in a decrease in the average linking number of plasmid DNA. A decrease in the superhelicity of chromosomal DNA of *E. coli* expressing Hc1 is reflected in the differential expression of the outer membrane porin proteins OmpC and OmpF. Analysis of *lacZ* reporters fused to supercoiling sensitive promoters also suggest a net relaxation of chromosomal DNA at low levels of Hc1 expression. Detailed biophysical analysis of Hc1-DNA interaction, *in vitro*, indicate that Hc1 preferentially binds supercoiled DNA. These results imply that Hc1 selectively stabilizes DNA of specific superhelical densities to modify DNA topology.

A nitrocellulose filter-binding assay has been used to characterize the cooperative binding of Hc1 to DNA and to examine the effects of pH, reducing conditions, and ionic strength on the interaction. Ionic strength had a marked influence on both DNA-binding and DNA-dependent aggregation of the protein. Reversibility of this aggregation was also ionic strength dependent with a discrete optimum ionic strength for maintaining stable association. The protein was found to migrate with an apparent molecular mass of 42-kDa by gel-filtration but behaved in a manner suggestive of a partially extended conformation. Hc1 associates with either positively or negatively supercoiled DNA in preference to relaxed DNA implying that the protein has the ability to discriminate between DNA domains of differing topology *in vivo*.

Since we suspect that any regulatory effects of chlamydial Hc1 are likely a consequence of its modification of DNA structure, our emphasis for the near term is upon detailed biochemical analysis of DNA-protein and protein-protein interactions in conferring higher-order chromatin structure. These *in vitro* studies incorporate a variety of established methods for analysis of DNA-protein interactions. We have developed a rapid and novel protocol for isolation of the EB nucleoid that appears to maintain its native conformation. Briefly, this consists of a two step procedure. The first step selectively solubilizes cytoplasmic components and non-structural outer membrane proteins and the lipopolysaccharide. This effectively leaves a "cage" of disulfide-linked Major Outer Membrane Protein enclosing an intact nucleoid. The second step consists of the addition of reducing agents to disrupt the crosslinked MOMP, leaving an intact nucleoid that maintains its native (condensed) conformation.





*Coxiella burnetii*, the etiologic agent of Q fever, undergoes a similar but less well defined developmental cycle involving metabolically inactive small-cell-variants (SCV) and metabolically active large-cell-variants (LCV). Although ultrastructural differences between the cell types have been clearly documented, little is known at the molecular level about *C. burnetii* morphogenesis. To initiate studies on the *C. burnetii* life cycle we purified SCV and LCV by cesium chloride equilibrium density centrifugation. Subsequent analysis of the purified cell types by SDS-PAGE and silver staining revealed a number of proteins unique to each cell type. We have identified in *C. burnetii*, histone H1 homologs of approximately 22 (Hq1) and 16 (Hq2) kDa. Like the chlamydial histone homologs, these highly basic proteins likely play a role in the chromatin condensation witnessed in the SCV and their synthesis is presumably developmentally regulated. Hq1 is primarily associated with the SCV. Preparative isoelectric focusing was employed to purify other basic proteins possibly involved in SCV chromatin structure. This led to the identification and cloning of a gene encoding a small, very basic protein of 35 amino acids (3.5 kDa) termed ScvA. Using antiserum raised against ScvA we demonstrated by western and immunoelectron microscopy that the protein is exclusively associated with the SCV. A peripheral labeling profile suggests that ScvA may be a structural component of the SCV outer surface. ScvA antiserum will be used to investigate the kinetics of ScvA synthesis and degradation by purified acid-activated *C. burnetii* and by organisms growing intracellularly. 2-D analysis of LCV and SCV proteins will be conducted and those shown to be differentially synthesized will be further characterized. Development of a synchronous infection model will be pursued which will allow analysis, at the mRNA and protein synthesis level, of programmed gene expression that occurs during the *C. burnetii* developmental cycle.

### Proposed Course of Work:

• An essential question is whether chlamydial Hc1 and Hc2 bind DNA cooperatively in mixed oligomers in a fashion roughly analogous to eukaryotic histones in the formation of nucleosomes or whether they are segregated in binding to regions of different composition or structure. The implications of having different domains of the chromosome under different constraints would be that certain regions of the chromosome might be more readily accessible to RNA polymerase for early transcription of specific genes during the initial stages of EB to RB transition. Conversely, unique regions of the chromosome might contain domains encoding some of the last genes expressed during the condensation of RBs to EBs.

With the availability of a rapid method to obtain high-quality EB nucleoids, it is now possible to initiate experiments to analyze how the two histone-like proteins are involved in the condensation seen in the chromatin of the EB nucleoid. Immuno-electron microscopy may be useful in this regard. This type of analysis will be complemented by the use of limited proteolysis and nucleolysis to fractionate chromatin domains that could be enriched on either Hc1 or Hc2. The strength of these interactions can also be analyzed by selective elution of Hc1 and Hc2 with increasing amounts of salt. Preliminary data suggests that, in fact, Hc1 and Hc2 can be differentially removed from the intact nucleoid, under relatively physiological salt conditions. The resulting altered nucleoid can then be analyzed for changes in structural integrity due to the specific loss of Hc1 or Hc2.

Longer term goals will address some obvious remaining questions. What environmental signals trigger histone synthesis and/or aggregation? Conversely, how are the histones released from DNA at the initial stages of the developmental cycle? DNA-protein interactions as a rule are reversible thus there are a number of potential models to consider in designing experiments to explore this important question. An important clue may be provided by *in vitro* studies. Aggregation of Hc1-DNA complexes is extraordinarily sensitive to ionic strength over a range that are reasonable in the bacterial cytoplasm. Study of this possibility will involve confirmation of disaggregation *in vitro* and establishing conditions to modify intracellular ions in the recombinant system that might dissociate the condensed nucleoid.





**Publications:**

Barry, C.E., III, Brickman, T.B., and Hackstadt, T., Regulation of Chlamydial Development by Hc1-Mediated Effects on DNA Structure. *Mol Microbiol* 1993;9:273-283.

Brickman, T.J., Barry, C.E., III, and Hackstadt, T., Molecular Cloning and Expression of *hctB* Encoding a Strain-Variant Chlamydial Histone-Like Protein with DNA-Binding Activity. *J Bacteriol* 1993;175:4274-4281.

Hackstadt, T., Brickman, T.J., Barry, C.E., III, and Sager, J., Diversity in the *Chlamydia trachomatis* histone homolog Hc2. *Gene* 1993;132:137-141.



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>PROJECT NUMBER</b> ZO1 AI 00672-02 LICP
<b>PERIOD COVERED</b> October 1, 1993 to September 30, 1994		
<b>TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)</b> Mucosal Immunity to Chlamydial Infection		
<b>PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)</b>		
PI:	H.D. Caldwell	Chief LICP, NIAID
Others:	T. Cotter	IRTA LICP, NIAID
<b>COOPERATING UNITS (if any)</b>  		
<b>LAB/BRANCH</b> Laboratory of Intracellular Parasites, Hamilton, Montana 59840		
<b>SECTION</b> Immunology of Intracellular Parasites		
<b>INSTITUTE AND LOCATION</b> NIAID, NIH, Bethesda, MD 20892		
<b>TOTAL MAN-YEARS:</b> ~ 1.5	<b>PROFESSIONAL:</b> 1.5	<b>OTHER:</b> 0
<b>CHECK APPROPRIATE BOX(ES)</b> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
<b>SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided..)</b>  <p>             The goal of this project is to directly determine whether Chlamydia specific secretory IgA (sIgA) plays a role in protection from, or resolution of, chlamydial infection of the mouse genital tract. In order to address this question we will 1) produce murine hybridomas that secrete polymeric IgA (pIgA) monoclonal antibodies (mAbs) specific to chlamydial surface components, and 2) determine their ability to protect against chlamydial genital tract infection in a murine model. mAbs will be administered to naive animals by subcutaneously implanting a mAb secreting hybridoma in the backs of syngeneic mice (backpack tumor system). This approach is made possible by the fact that systemically delivered pIgA is specifically complexed with secretory piece and transported to mucosal surfaces as sIgA, whereas monomeric IgA (mIgA) and IgG are not. To date, three murine hybridomas have been generated that produce pIgA specific for the surface of <i>Chlamydia trachomatis</i> MoPn. These hybridomas resulted from fusion experiments using mesenteric lymph node derived lymphocyte populations that were enriched for IgA surface positive cells. Using pIgA and IgG secreting hybridomas, we are currently conducting a pilot experiment to characterize the backpack tumor system in our laboratory. Preliminary data indicate that the take rate for tumor development is &gt;80%, that high levels of mAb are produced by such tumors, and that tumor growth rates vary significantly between tumor cell lines. With this information, we can now evaluate the role of anti-chlamydial sIgA in protection against chlamydial colonization of the genital tract mucosae as well as its role in resolving established infections of the genital tract. The same collection of mAbs will allow for future studies that compare <i>in vivo</i> protection with <i>in vitro</i> neutralization of chlamydial infectivity, as well as, investigations of mucosae specific IgA antibody dependent cellular cytotoxicity as a potential mechanism of protective immunity.           </p>		



## Background and Objectives:

*C. trachomatis* is an obligate intracellular prokaryotic pathogen that primarily colonizes the oculogenital tract mucosa. In humans, *C. trachomatis* infections are a major cause of bacterial STDs for which a vaccine is badly needed. The development of animal models to study mucosal immunity to chlamydial infection are key to understanding immune mechanisms that function in protective immunity. The objective of this project is to use a murine model of chlamydial genital tract infection to define and characterize the protective immune responses that develop following chlamydial infection. Specifically, we will address the contributions of sIgA and mucosal cell mediated immunity (CMI) in protective immunity.

## Major Findings:

A murine model of chlamydial genital tract infection is being used for these studies. Ideally, it would be desirable to study mucosal immunity in this model using lymphocytes obtained from inductive and effector sites of the genital tract mucosa. However, this is not possible because the inductive sites of mucosal immunity in the murine genital tract are not well defined, and sufficient numbers of lymphocytes cannot be readily isolated from effector tissue sites (sub-epithelial and epithelial cells) of the genital tract for immunological studies at the cellular level. We have found that infection of the mouse gastrointestinal epithelia with the *C. trachomatis* murine biovar (MoPn) confers protective immunity to subsequent vaginal challenge; demonstrating that the immune responses produced by gut associated lymphoid tissue (GALT) are relevant for the study of protective immunity in the mouse genital tract. This is an important finding because the features of the inductive and effector sites of the GALT are well characterized, and as importantly, sufficient numbers of lymphocytes can be isolated from these sites for conducting immunological studies at the cellular level. We have used ELISPOT to enumerate the number of chlamydial specific sIgA plasma cells in the Peyer's patches (PP) and mesenteric lymph nodes (MLN) at various times following gut infection of Balb/c mice with MoPn. The peak response of chlamydial specific sIgA positive plasma cells was in the MLNs at 10-14 days post infection. Standard polyethylene glycol mediated fusions were performed using total MLN lymphocytes enriched for IgA surface positive B-cells by depleting T-cells, and IgG and IgM surface positive B-cells. Using this methodology we have generated hybridomas that produce pIgA specific to surface accessible epitopes of the MoPn major outer membrane protein (MOMP). Previous attempts to identify chlamydial specific cytotoxic lymphocytes have been unsuccessful. These failures could be explained by the use of inappropriate effector and target cell populations in CTL assays. Studies using intraepithelial lymphocytes (IELs) from chlamydial infected mucosal tissue in combination with chlamydial infected epithelial cell targets have not been done. This combination may indeed be critical, as it is possible that specific T-cell subsets, such as  $\gamma/\delta$  T-cells may function as cytotoxic T-cells and their ability to recognize chlamydial infected cells might be restricted in tissue recognition (e.g., epithelial cells). Preliminary to this work, methods have been developed to obtain purified populations of lymphocytes from both the lamina propria and gut epithelia. Phenotypic analysis of T-cells isolated from these tissues found that immune mice have enriched populations of CD4<sup>+</sup> T-cells in the lamina propria, and CD8<sup>+</sup> and  $\gamma/\delta$  TCR<sup>+</sup> T-cells in the gut epithelia.

We have identified two chlamydial protein antigens (22 and 34 kDa) which were detected by immunoblotting using convalescent immune sera. The 22 and 34 kDa proteins were not present in purified chlamydial developmental forms but were readily detected in lysates of chlamydial infected cells. Immunofluorescence staining of chlamydial infected cells with convalescent antisera absorbed with intact chlamydiae showed a diffuse pattern of immunoreactivity within the chlamydial inclusion and surrounding inclusion membrane. These findings indicate that the 22 and 34 kDa antigens may be proteins that are secreted by chlamydiae during intracellular growth which associate with the chlamydial inclusion membrane.





## Proposed Course of Work:

The *in vitro* neutralizing activity of plgA mAbs specific to MOMP will be determined and compared to anti-MOMP IgG neutralizing mAbs. The *in vitro* neutralization characteristics of plgA may be important correlates to the ability of these mAbs to protect *in vivo*. We will assess the ability of plgA to protect *in vivo* by two different methods. Affinity purified anti-MOMP plgA and MOMP specific IgG mAbs, will be administered to naive mice via intravenous injection or by subcutaneously implanting the mAb secreting hybridoma in the mouse back. The levels of chlamydial specific mAbs secreted from blood to the cervicovaginal mucosa will be determined by ELISA. If serum anti-chlamydial plgA is secreted into the mouse vagina its protective efficacy will be assayed by intra-vaginal chlamydial challenge. Challenge protocols will be used to evaluate the ability of anti-MOMP plgA to protect against chlamydial adsorption, colonization, and growth within the vaginal epithelia. Lymphocytes isolated from gut lamina propria and epithelia from immune mice will be used as effector cells in both lymphocyte proliferative and cytotoxic assays using H-2 compatible chlamydial infected gut epithelial derived target cells. If specific lymphoproliferative or cytotoxic responses are found functional T-cells subset(s) will be identified by specific depletion of lymphocyte subsets with mAbs and by cytokine profiles. If these experiments yield positive results CTL clones will be generated and characterized in regard to cell surface phenotype, cytokine profiles, and antigen specificity. T-cell clones will be used to identify chlamydial peptides(s) presented by MHC class I molecules by conventional peptide sequencing techniques. Because chlamydiae are confined within a cytoplasmic vacuole throughout their intracellular growth cycle, and are not known to secrete molecules into the cytosol, it is possible that classical endogenous antigen processing and presentation to class I molecules in the cytosol does not occur in chlamydial infected cells. Recent studies have shown that the nonconventional murine class I-b molecules play a specialized role in prokaryotic host defenses through specific binding and presentation of N-formylated bacterial peptides to cytotoxic T-cells. We will investigate whether this non-classical mechanism might function in the generation of chlamydial specific CTLs.

We will clone and sequence the genes encoding the 22 and 34 kDa proteins that have been implicated as possible "secreted chlamydial proteins." Recombinant bacteriophage libraries will be generated and antigen-producing clones identified by immunologic screening with absorbed convalescent antisera. Genes will be cloned into expression/purification systems and the purified recombinant proteins will be used to produce monospecific antisera which will then be used to localize the proteins in chlamydial infected cells by immunoelectron microscopy. If one or more of these proteins are found to be associated with the inclusion membrane, cross-linking experiments using bi-functional reagents will be done to examine *in situ* associations of the protein(s) with the inclusion membrane. Additional studies will include the study of cytotoxic and other immune responses which may be stimulated by these proteins.

## Publications:

New Project, under study, presently no publications.





<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>PROJECT NUMBER</b> ZO1 AI 00693-02 LICP
<b>PERIOD COVERED</b> October 1, 1993 to September 30, 1994		
<b>TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)</b> Pathogenic Mechanisms of <i>Mycobacterium tuberculosis</i>		
<b>PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)</b>		
PI:	C.E. Barry	Unit Head LICP, NIAID
Others:	Y. Yuan S. Stewart	Visiting Associate Microbiologist LICP, NIAID LICP, NIAID
<b>COOPERATING UNITS (if any)</b> None		
<b>LAB/BRANCH</b> Laboratory of Intracellular Parasites, Hamilton, Montana 59840		
<b>SECTION</b> Mycobacterial Unit		
<b>INSTITUTE AND LOCATION</b> NIAID, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2	.5
<b>CHECK APPROPRIATE BOX(ES)</b> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
<b>SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided..)</b>  <p>The overall objective of this work is the development of novel chemotherapeutics and chemotherapeutic targets for the treatment of diseases of Mycobacterial origin including tuberculosis, leprosy, and <i>M. avium-intracellulare</i> (MAC) infections of AIDS patients. Pathogenic strains of mycobacteria uniformly modify their major mycolic acids by cyclopropanation while non-pathogenic strains do not. We have used this distinction to clone a gene from a pathogenic strain (<i>M. tuberculosis</i>) into a non-pathogenic strain (<i>M. smegmatis</i>) which cyclopropanates mycolic acids. By doing this we have created and characterized a novel mycolic acid which is composed of a hybrid between the normal <i>M. smegmatis</i> and <i>M. tuberculosis</i> major mycolic acids. Identification of the gene involved revealed that the encoded protein has high homology to the only known enzyme with a related function, the <i>E. coli</i> cyclopropane fatty acid synthase. In addition, sequencing the genomic DNA flanking this gene has revealed the presence of two new proteins which appear to be homologous to genes which would be involved in lipid biosynthesis. Thus we believe that we have cloned a cluster of biosynthetic genes which are related to mycolic acid biosynthesis. Each of these genes independently represents a potential drug target. We have also synthesized a novel class of inhibitors targeted towards cyclopropanated mycolic acid biosynthesis which are based on sulfur-substituted fatty acid derivatives and shown that these compounds selectively inhibit the growth of <i>M. tuberculosis</i>.</p>		



## Background and Objectives:

The WHO estimates that 30 million people will die from tuberculosis in the next decade without immediate action. In developed countries, the recent appearance of multiply drug resistant (MDR) tuberculosis in the AIDS community is particularly alarming. 90% of MDR tuberculosis cases have occurred in HIV-positive patients with mortality rates of 70-90% and only 4-16 weeks between diagnosis and death. It is clear that current chemotherapy for tuberculosis is inadequate and new drugs are desperately needed. One of the major targets of current anti-mycobacterial drugs is biosynthesis of outer cell-wall components, in particular, the mycolic acids. This biosynthetic pathway is the target of isoniazid, ethionamide, thiocarlide, and possibly ethambutol. In spite of the importance of this pathway as a chemotherapeutic target, not a single enzyme activity or gene has been identified which is directly involved in the biosynthesis of mycolic acids.

## Major Findings:

We have utilized the ability to transform *M. smegmatis* to localize at least one biosynthetic gene directly involved in mycolic acid biosynthesis from *Mycobacterium tuberculosis*. Slow-growing species of mycobacteria (including *M. leprae*, *M. tuberculosis*, *M. ulcerans*, *M. avium*, and others) uniformly modify their major mycolic acids at two positions by enzymatically transforming a *cis* double bond into a cyclopropane ring while fast-growing, non-pathogenic strains do not. We constructed a cosmid library from *M. tuberculosis* genomic DNA in an *E. coli*-mycobacterium shuttle vector and transformed *M. smegmatis* with this library. Recombinant clones were screened for cyclopropanation of their mycolic acids by purifying total mycolates and examining them on a thin-layer chromatography system which selectively retards components containing *cis* double bonds. Using this screening method we examined the mycolates from over 600 cosmid-containing clones and discovered two with modified mycolates. The modified mycolates have been purified to homogeneity and their structure has been deduced based upon NMR and mass spectral analysis. This novel mycolate was shown to consist of the normal *M. smegmatis* major-mycolate with an added cyclopropane at the predicted position. Isolation of the DNA from these two clones revealed that they were overlapping, non-identical cosmids containing 30-35kb inserts. Shotgun cloning of fragments from these cosmids localized the enzyme activity to a shared 7.2kb BamHI fragment of which we have currently sequenced about 6kb. In the newly sequenced region we have discovered an open reading frame of 864 nucleotides (*cma*) which codes for a protein of 288 amino acids and displays a high degree of homology to the *E. coli* cyclopropane fatty acid synthase - the only characterized enzyme which catalyzes the formation of a cyclopropane ring. The *cma* gene alone has been shown to be sufficient to convert *M. smegmatis* mycolates to the cyclopropanated type. In addition to the cyclopropanating enzyme, we have discovered two additional ORFs which appear to be utilized (as determined by GC bias in the third codon position as well as Fickett rare codon analysis). The predicted protein product of ORF2 is highly homologous to a group consisting of reductases and dehydrogenases which function in lipid biosynthesis from a diverse set of organisms. We have also identified a third ORF whose protein product is apparently related to a hydratase/dehydratase or dehydrogenase from *Candida tropicalis* which could also function in mycolate biosynthesis. These enzymes may be components of a unique kind of Type II fatty acid synthase involved in mycolate production or in the late stages of mycolate biosynthesis in *M. tuberculosis* and offer strong evidence that we are within an operon encoding multiple enzyme activities related to mycolic acid biosynthesis. Since each enzyme activity involved is unique to mycobacteria, each represents a potential drug development target if we can assess their *in vivo* function and develop *in vitro* assays. A related ongoing project illustrates the potential of this information. In the biosynthesis of mycolates a 24 carbon fatty acid is specifically desaturated and then extended before cyclopropanation. We designed a series of isosteric compounds which should be extended normally but which contain a sulfur atom at the position which is ultimately cyclopropanated. When tested *in vitro* against growing *Mycobacterium tuberculosis* these compounds displayed a potent anti-bacterial activity with MIC<sub>50</sub>s of the best of these approaching that of isoniazid (1 µg/ml of 4-Thiatetracosanoate). Importantly, *M. smegmatis*, which does not cyclopropanate its mycolic acids, is unaffected by these drugs. We are continuing our work on these compounds *in vitro* and have synthesized radiolabelled 6-thiatetracosanoate. This compound has allowed us to demonstrate



that radiolabelled mycolate precursors in fact enter *M. tuberculosis* and are elongated as predicted to meromycolates of about 36 carbons in length which then block further synthesis of mycolic acids. In complementary experiments we have demonstrated an accumulation of 26-carbon intermediates using radiolabelled acetate and unlabelled thiatetracosanoates.

### **Proposed Course of Work:**

During the next year this program will focus on three areas; (1) We will continue to sequence and analyze the DNA regions contiguous to the cyclopropane synthase activity in the hopes of uncovering further mycolic acid biosynthetic genes. In particular we will utilize the cosmid sequences we presently have to continue into adjacent regions and design primers to lead into linked cosmids. (2) We will further analyze both the genetics and the biochemistry of the cyclopropane synthase activity. This is especially critical as a tool to understand the global regulation of mycolate biosynthesis. We will map the transcriptional start site and define promoter elements of this and related genes. We will construct CAT fusions and analyze growth-phase and environmental condition dependent regulation of this gene in *vivo* models of mycobacterial infection (including macrophages). We will attempt to demonstrate the immediate precursor and product of this enzymatic transformation and elucidate any required cofactors in the enzymatic reaction. (3) We will attempt to design and synthesize new and improved versions of the thiatetracosanoates as well as carry these compounds through some preliminary toxicity and efficacy studies. These will be done, in part, in conjunction with a CRADA agreement which is currently being negotiated with PathoGenesis Corporation of Seattle.

### **Patents:**

Barry, C.E., Yuan, Y. US Patent 08/210,519: Novel Anti-Mycobacterial Compositions and their use for the Treatment of Tuberculosis and Related Diseases, Pending.

### **Publications:**

New project under study, presently no publications.



**LABORATORY OF MICROBIAL STRUCTURE AND FUNCTION**  
**Rocky Mountain Laboratories**  
**Hamilton, Montana**  
**1994 Annual Report**  
**Table of Contents**

<b>Z01 AI</b>		
<b>Project Number</b>		<b>Page</b>
Summary		16-1
00193-15	Gonococcal Surface Components: Structure and Function -- Swanson	16-7
00480-09	Pathogen-Arthropod Interactions of Vector-borne Diseases Affecting Public Health -- Schwan (transferred from LVP)	16-8
00492-08	Molecular Basis for Infection by <i>Borrelia burgdorferi</i> -- Schwan (transferred from LVP)	16-9
00516-07	Immunoglobulin Biology -- Pincus	16-10
00549-06	Molecular Mechanisms of Variation and Adaptation in <i>Borrelia burgdorferi</i> -- Rosa	16-11
00568-05	Molecular Basis and Extent of Microbial Sex -- Heinemann	16-12
00608-04	Expression and Phase Variation of Gonococcal <i>opa</i> Genes -- Belland	16-13
00609-03	Synthetic Gene Approach to Study the Action of Anti-bacterial Genes of Mammals -- Bhat (terminated)	16-14
00632-03	Siderophores and Iron Transport in <i>Pseudomonas aeruginosa</i> -- Ankenbauer (terminated)	16-15
00633-02	Neisserial-Host Interactions: Pathogenic Mechanisms and Host Responses -- Fischer (terminated)	16-16
00694-01	Genetic Analysis of the Gonococcus -- Hill	16-17







Annual Report  
LABORATORY OF MICROBIAL STRUCTURE AND FUNCTION  
Rocky Mountain Laboratories  
Hamilton, Montana  
National Institute of Allergy and Infectious Diseases  
October 1, 1993, to September 30, 1994

**RESEARCH HIGHLIGHTS**

The Laboratory of Microbial Structure and Function researches several microbial pathogens that cause human diseases, including *Neisseria gonorrhoeae* (gonorrhea), *Borrelia burgdorferi* (Lyme disease), *Borrelia hermsii* (relapsing fever), *Yersinia pestis* (bubonic plague), group B streptococci (neonatal meningitis, septicemia) and human immunodeficiency virus (HIV, AIDS). A major thrust of all those studies is variation in microbial surface components that have roles in pathogenesis or virulence, including its genetic basis, biochemical accompaniments, and immunological consequences.

Tom Schwan and Patti Rosa found that *B. burgdorferi* (the causative agent of Lyme disease) will express its OspC outer surface protein *in vitro* when culture temperature is shifted from 24 to 37°C; the reverse temperature shift shut-off OspC production. *B. burgdorferi* infecting the midgut of an unfed tick are OspC-, but they become OspC+ during three day blood meals; such prolonged feeding periods by infected ticks efficiently transmit spirochetes to mammals. Organisms that infect mammals appear to express OspC, as deduced from the anti-OspC antibody response that appears. This demonstration that Lyme disease spirochetes change their surface constitutions during residence in ticks provides important insight into the relationships of these bacteria to their mammalian and tick hosts and in their transmission between hosts; it is a first demonstration that bacterial pathogens alter major surface constituents while residing in arthropod vectors.

Patti Rosa has previously documented recombinational rearrangements in the *ospA* and *ospB* genes of *B. burgdorferi* leading to antigenically variant OspA and OspB phenotypes. Rosa and Neil Margolis recently identified a OspA- OspB- OspC+ phenotype in which transcriptional regulation was implicated in lack of *ospA/B* expression. Cloning and sequencing of the *ospC* gene by Margolis was accompanied by finding upstream genes resembling guanine synthesis-encoding elements of *E. coli*. It appears likely that expression of OspC and these purine biosynthetic enzymes relate to life of spirochetes in their tick vector versus mammalian hosts which constitute vastly dissimilar environments differing widely in guanine levels (high in ticks, low in mammal blood). Homologues of the purine synthesis genes were located in *B. hermsii* on a linear plasmid - the first demonstration of biosynthetic genes on such structures. It was also shown that the *ospC* gene of *B. burgdorferi* has high homology to *B. hermsii* genes encoding the large family of "variable membrane proteins" (Vmp's) whose expressions correlate with relapses in relapsing fever. Kit Tilly identified *B. burgdorferi* genes that complement HU and IHF functions of *E. coli*. Tilly also identified a *rho* and *rpsT* homologues in *B. burgdorferi*.

Studies of interactions between *Y. pestis* (bacteria causing bubonic plague) and their Oriental rat flea vectors (Joseph Hinnebusch and Tom Schwan) are now possible due to their having developed and adapted several techniques enabling detection and quantitation of *Y. pestis* in individual fleas. Application of these newly-devised methods, along with the ability to introduce *Y. pestis* into fleas that



feed across mouse skin *in vitro*, is just underway to define *Y. pestis* traits that are related to, and perhaps essential to, survival of these bacterial pathogens in fleas.

Jack Heinemann continued his work on microbial sex as he and Bob Ankenbauer demonstrated that bacteria that have been rendered non-viable by several antibiotics remain potent as conjugal donors of their genes, including some that encode antibiotic resistance, to other bacteria. Heinemann and John Klena have explored some surface requirements for bacteria engaged in conjugation; they find that different "deep-rough" LPS mutants are compromised as donors, recipients, or both.

Iron acquisition by *Pseudomonas aeruginosa* was studied by Bob Ankenbauer and Cheryl Dooley who identified several genes involved in the synthesis of the pyoverdinin and pyochelin siderophores. Ankenbauer identified, cloned, and sequenced the major Fe-pyochelin outer membrane receptor of *pseudomonas*; unexpectedly, this molecule showed strong similarity to receptors for iron siderophores in the hydroxamate class, of which pyochelin is not a member.

Seth Pincus compared serum anti-HIV antibodies of vaccinated individuals, accidentally-infected lab workers, and chimpanzees that were immunized and then experimentally-infected, and found striking differences in their specificities. The three groups generate antibodies that recognize quite different portions of gp120 and gp41 moieties of HIV. Antibodies in the infected lab workers demonstrate that the V1-loop of gp120 is a previously-unappreciated immunodominant target of neutralizing antibodies. Immunotoxins previously constructed by Pincus were used to select 1) lymphocyte host cells incapable of propagating infectious HIV and 2) HIV variants. The immunotoxin-resistant lymphocytes that produce no HIV are being studied by Pincus and Hua Fang; one such cell is infected with HIV whose genome harbors an insertion mutation near the left LTR representing part of a host cell lysine-tRNA gene. Immunotoxin-resistant HIV variants have been studied by Tom Duensing and Pincus, and mainly represent mutations that alter antigenicity in gp160, impair viral processing, or both. New linkers to cross-link antibody and toxic moieties into immunotoxins are being designed and synthesized by Vladimir Tolstikov. These include an oligopeptide that is uniquely susceptible to cleavage by HIV protease in order to target immunotoxins even more precisely to HIV-infected cells; another linker contains part of the sequence of the influenza hemagglutinin, utilized to disrupt lysosomes and enhance entry of the immunotoxin into the target cell cytoplasm.

Recent work by Pincus on his previously-described opaque and transparent colonial variant group B streptococci (GBS) shows that both variants occur in clinical specimens; this was previously unappreciated because isolation media in general use do not support growth of some variants. The GBS colonial variants exhibit distinctive interactions with human neutrophils *in vitro* and display differing virulence in laboratory animal infection models.

Studies on *N. gonorrhoeae* proceeded in several interconnected directions, including examination of selected physicochemical and biological characteristics of the organism's surface and elucidating control mechanisms for gonococcal surface components. Bob Belland found that several genes influence the "non-homologous recombination" (NHR) events involved in phase switching the expression of several gonococcal components including Opa proteins, PilC, lipooligosaccharide, and a restriction/modification system (*ngoX*) that Belland has recently discovered. Factors that have been demonstrated to influence NHR frequencies in gonococci include mismatch repair enzymes (MutS and MutL), the degree of DNA supercoiling, and levels of transcription. Belland previously introduced



recombinant *opa* genes into *E. coli* that then behaved toward tissue culture cells and neutrophils *in vitro* like gonococci expressing the same Opa protein; but mosaic *opa* genes with part of one *opa* replacing the analogous region of another *opa*, though expressed in *E. coli*, did not endow them with surface behavior like either Opa on incubation with eukaryotic cells. Studies by Tie Chen established that Opa<sup>+</sup> gonococci adhere to eukaryotic cells in tissue culture via heparin-like surface glycosaminoglycans; the avid adherence of Opa<sup>+</sup> organisms was inhibited by heparin and DNA but not by several other polyanions (chondroitin sulfate, others). It is provocative that polyanions also have striking influences on the adherence and infectivity of several other sexually-transmitted microbes including chlamydiae, herpes simplex virus, and HIV. Perhaps that phenomenon will be useful for development of chemical barriers against STD-causing microbes. Particular Opa<sup>+</sup> gonococci avidly adhere to a wide variety of eukaryotic cells, including non-human cells; but pilus-mediated gonococcal adherence is specific for human cells. Chen has also demonstrated that incubation of pilus<sup>+</sup> Opa<sup>+</sup> gonococci in urine renders them resistant to killing by normal human serum; however this effect was not demonstrable with all urines - even from the same individual - and its molecular origins are unclear. Stuart Hill returned to continue studying pilus genetics and DNA transformation of gonococci. He found that, although Opa<sup>-</sup> organisms are more competent for transformation, Opa<sup>+</sup> cells are more efficient in being transformed. Hill and Belland cloned gonococcal genes that encode the two subunits (Him, Hip) of "integration host factor" (IHF) which bends DNA and is involved in homologous, recA dependant recombination; Hill has recently purified these subunits. John Carlson has successfully constructed recombinant fusion genes containing *opa* control regions and reporter molecules to examine the role of *opa* transcription on phase variation. John Swanson has correlated lipooligosaccharide (LOS) side chain structure, cell surface net charge, hydrophobicity, and sensitivity to the  $\alpha$ -helix forming amphipathic peptide magainin-2. Swanson also examined the influences of Opa and pilus expression on gonococcal surface charge, demonstrating that some combinations of LOS, Opa, and pilus phenotypes endow these organisms with positive charge. In collaboration with Kim Wise (University of Missouri), the variable lipoprotein of *Mycoplasma hyorhinitis* was found to influence surface charge of this organism. John Klena undertook identification of gonococcal genes involved in LOS synthesis; in the process he isolated an *E. coli* gene involved in early stages of lipopolysaccharide core synthesis.





## **ADMINISTRATIVE REPORT**

Personnel changes in LMSF during FY'93 include the recruitment of Brian Stevenson (State University of New York at Stony Brook) Yale University School of Medicine, and Mark Niebylski (University of Notre Dame), as IRTAs, and Stuart Hill (University of Montana) University of Oregon and formerly LMSF, as Staff Fellow. Tom Schwan (Senior Staff Fellow), Joe Hinnebusch (IRTA), and technical staff R. Karstens and M. Schrupf were transferred to LMSF when the Laboratory of Vectors and Pathogens was changed to RML Microscopy Branch. Summer IRTA students were Heidi Scott (Marlboro College), John Fuhrman II (Norfolk High School), Tara Wehrly (Washington University), and Lynn Race (Montana State University). Seminars were given by a number of outside guests: Arthur Krieg (University of Iowa), Tone Tonjum (National Hospital, University of Oslo), Michael Koomey (University of Michigan Medical School), Philippe Anker (University of Geneva), Soji Bitoh (University of Manitoba), Colin Tinsley (Rockefeller University), Kim Wise (University of Missouri-Columbia), Valentin Rybchin (State Technical University, St. Petersburg, Russia), Mirosław Gorny (New York University Medical Center), Paul Ewald (Amherst College), Gregory Russell (Laboratory of Parasitic Diseases, NIAID), Donald Champagne (University of Arizona), Lise Gern (University of Neuchatel), and Dennis Kopecko (Laboratory of Enteric and Sexually Transmitted Diseases, FDA).





## HONORS AND AWARDS

### Journal Editorial Boards:

- S. Pincus - Medical advisory board for Arthritis Today  
T. Schwan - Journal of Clinical Microbiology

Manuscripts were reviewed by LMSF staff for the following journals: American Journal of Tropical Medicine and Hygiene, Applied and Environmental Microbiology, Canadian Journal of Microbiology, Cell, Clinical Infectious Diseases, Infection and Immunity, Journal of Bacteriology, Journal of Biological Chemistry, Journal of Clinical Investigation, Journal of Clinical Microbiology, Journal of Experimental Medicine, Journal of General Microbiology, Journal of Immunology, Journal of Infectious Diseases, Journal of Molecular Biology, Journal of Rheumatology, Journal of Spirochetal and Tick-Borne Diseases, Microbial Pathogenesis, Molecular Microbiology, New England Journal of Medicine, Plasmid, Proceedings of the National Academy of Sciences USA, Protein Expression and Purification, Science, and Trends in Microbiology.

### Professional Posts:

- J. Heinemann - Affiliate Faculty, University of Montana, Missoula, MT  
S. Pincus - Adjunct Associate Professor of Internal Medicine, University of Utah,  
Salt Lake City, UT

### Invited Lectures and Participation in Meetings and Symposia:

- R. Belland - 94th Meeting American Society for Microbiology, Las Vegas, NV  
ASM Northwest Branch/CSM Western Branch Meeting, Victoria, B.C. Canada  
9th International Pathogenic Neisseria Conference, Winchester, England
- T. Duensing - 94 UCLA/UCI AIDS Symposium, Palm Springs, CA
- H. Fang - 1st National Conference on Human Retroviruses and Related Infections,  
Washington, DC
- J. Heinemann - Abbott Laboratories, Abbott Park, IL  
University of South Florida, Tampa, FL  
University of Canterbury, Christchurch, New Zealand  
7th International Symposium on the Genetics of Industrial Microorganisms,  
Montreal, Quebec Canada  
Gordon Conference, Microbial Stress Response, Plymouth State College, NH
- S. Hill - 9th International Pathogenic Neisseria Conference, Winchester, England
- S. Pincus - 2nd Berlin International Workshop on Immunology and Clinical  
Management of Infection in Transportation, Berlin, Germany  
Seminar at ImmunoAG, Vienna, Austria  
6th Annual Meeting on the Development of AIDS Vaccines, Alexandria, VA  
7th International Conference on Anti-Viral Research, Charleston, SC  
Keystone Symposium on Antibody Engineering, Lake Tahoe, CA
- P. Rosa - International Conference on Zoonoses, Piestany, Slovak Republic  
Gordon Research Conference, Biology of Spirochetes, Ventura, CA  
VI International Conference on Lyme Borreliosis, Bologna, Italy



- T. Schwan - Gordon Research Conference, Biology of Spirochetes, Ventura, CA  
University of Arizona, Tucson, AZ  
University of Montana, Missoula, MT  
Discussion leader, VI International Conference on Lyme Borreliosis,  
Bologna, Italy
- J. Swanson - 94th Keystone Symposium on Molecular Events in Microbial Pathogenesis,  
Santa Fe, NM  
University of Texas Health Science Center at San Antonio, San Antonio, TX  
University of Montana, Missoula, MT  
International Symposium on Microbiology and Molecular Biology, Umeå,  
Sweden  
9th International Pathogenic Neisseria Conference, Winchester, England

#### **Other Activities:**

- R. Belland - NIAID, RML, Institutional Biosafety Committee
- S. Hill - NIAID, RML, Library Committee
- S. Pincus - State of Montana Advisory Committee for Institutional Development Award  
HIV Peer Review Panel for the American Institute of Biological Sciences,  
Washington, DC  
NIAID, RML, Institutional Biosafety Committee
- P. Rosa - *Ad hoc* reviewer, Molecular Biology/Genetics Study Section, Arthritis  
Foundation  
Technical reviewer, Lyme disease cooperative agreement proposals, Centers  
for Disease Control  
Reviewer, NSF/MONTS EPSCor program  
NIAID, RML, Animal Care and Use Committee
- T. Schwan - Elected Council Member, American Committee of Medical Entomology  
(1991-1995)  
Vice President, International Northwestern Conference on Diseases of Nature  
Communicable to Man  
NIAID, RML, Animal Care and Use Committee  
NIAID, RML, Safety Committee  
NIAID, RML, Library Committee
- J. Swanson - *Ad hoc* reviewer, Bacteriology and Mycology-1 Study Section, NIAID  
Review Panel for the Postdoctoral Research Fellowships for Physicians  
program, Howard Hughes Medical Institute, Chevy Chase, MD  
Reviewed Merit Review Application, Department of Veterans Affairs,  
Livermore, CA  
Reviewed National Research Council proposals to the Agency for  
International Development research grants program for the Historically  
Black Colleges and Universities, Washington, DC  
Dissertation Committee for the Ph.D. thesis of Blanca Restrepo, University of  
Texas Health Science Center at San Antonio, San Antonio, TX  
Chairman, NIAID, RML, Animal Care and Use Committee
- K. Tilly - *Ad hoc* reviewer for National Science Foundation grant  
NIAID, RML, EEO Committee



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00193-15 LMSF

PERIOD COVERED

October 1, 1993, to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genococcal Surface Components: Structure and Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	J. Swanson	Chief	LMSF, NIAID
Others:	J. Klena	IRTA Fellow	LMSF, NIAID
	J. Carlson	IRTA Fellow	LMSF, NIAID
	S. G. Morrison	Microbiologist	LMSF, NIAID
	J. M. Wilson	Biologist	LMSF, NIAID
	D. Hogan	Microbiologist	LMSF, NIAID

COOPERATING UNITS (if any)

E. C. Gotschlich (Rockefeller University) and K. Wise (University of Missouri)

LAB/BRANCH

Laboratory of Microbial Structure and Function

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

4.1

PROFESSIONAL:

2.5

OTHER:

1.6

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies in FY'94 on structure and function of gonococcal surface components were continuations of previously-initiated attempts to describe biological behaviors of these organisms to the physicochemical attributes of their surfaces. Earlier results on electrophoretic characteristics of whole gonococci established that both lipooligosaccharide (LOS) and Opa outer membrane proteins exert individual and collective influences on cell surface charge. Some Opa proteins contribute positive charge on whole gonococci. Other Opa proteins greatly enhanced negativity in spite of their having abundant cationic amino acids in their (predicted) surface-exposed portions. This affect was shown to result from accretion of polyanions (such as DNA and sulfated polysaccharide) on cells that expressed certain Opa proteins. These Opa+ gonococci, in effect, acquire a "garbage" capsule. Results also show that pilus+ cells are less negatively charged than their pilus- counterparts. Piliation 'dampens' influences of LOS, Opa, and polyanion accretion on cell charge, with pilus+ cells maintaining a net charge near zero, often slightly positive, regardless of the environment or which outer membrane components they express. Most recent studies indicate strict correlation between LOS side chain structure and gonococcal charge, with single sugar charges producing differences in whole cell electrophoretic mobilities A -2.1 to -0.2  $\mu\text{m-cm/V-S}$ . Gonococcal charge correlates with susceptibility to the oligopeptide magainin-2 which forms pores in bacterial membranes.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00480-09 LMSF

PERIOD COVERED

October 1, 1993, to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pathogen-arthropod Interactions of Vector-borne Diseases Affecting Public Health

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	T. G. Schwan	Microbiologist	LMSF, NIAID
Others:	J. Hinnebusch	IRTA Fellow	LMSF, NIAID
	M. Niebylski	IRTA Fellow	LMSF, NIAID
	R. H. Karstens	Bio Lab Tech (Micro)	LMSF, NIAID

COOPERATING UNITS (if any)

Ken Gage (CDC); Alan Barbour (UTHSC); Blanca Restrepo (UTHSC); Donald Anderson (Sacred Heart Hospital); Robert Perry (U Kentucky)

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

1.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objectives of this project are to use molecular and classical approaches to investigate pathogen-arthropod interactions of vector-borne agents causing diseases of human importance in the United States. Most of our effort has concentrated on *Yersinia pestis*, the causative agent of bubonic plague, and *Borrelia hermsii*, an agent of tick-borne relapsing fever.

Previously we described the use of the polymerase chain reaction (PCR) to rapidly detect and identify *Y. pestis* in fleas. This will assist in field surveillance when determining the risk of transmission of *Y. pestis* by fleas to humans in areas where plague is occurring. During the last year considerable effort has been directed towards the development of a quantitative, competitive PCR that will determine the number of *Y. pestis* cells in individual infected fleas. This assay is a prerequisite to our studies examining the blocking phenomenon in fleas of both the wild-type *Y. pestis* and various isogenic mutants. The quantitative, competitive PCR, or QPCR, involves primers that will amplify two target sequences of DNA during the same reaction, the wild-type target and the same region that has been shortened by an internal deletion. Both targets are amplified during the same PCR reaction, resulting in two amplification products, the larger product amplified from the wild-type sequence, and a smaller product from the deleted form of the same sequence. In the QPCR, the amount of the two amplification products will vary depending on the amount of the two target sequences available. For our QPCR to estimate the number of *Y. pestis* cells in individual fleas, we have chosen the *fur* (ferric iron uptake regulation) gene as the target. The amplification product of the wild-type sequence is 328 base pairs. A 65-base pair fragment was deleted from a recombinant clone containing the *fur* gene (original clone provided by Robert Perry, Univ. of Kentucky) and this DNA provided the competitive target sequence added to the QPCR. Therefore both a 328-base pair (wild type) and a 263-base pair (deletion mutant) amplification product should result when both target sequences are available in the reaction using the one set of primers. With a constant amount of the competitor added to a series of different concentrations of the wild-type DNA, a standard curve was generated using scanning laser densitometry of the film negative of the agarose gel containing both amplification products. Using this assay with Oriental rat fleas that were experimentally infected with *Y. pestis* in the laboratory, we were able to detect down to 100 bacteria and distinguish levels of infection in single fleas. This will allow us to determine the magnitude of infection in fleas that are infected with mutant *Y. pestis*.







DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00492-08 LMSF

PERIOD COVERED

October 1, 1993, to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Basis for Infection by *Borrelia burgdorferi*

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	T. G. Schwan	Microbiologist	LMSF, NIAID
Other:	J. Hinnebusch	IRTA Fellow	LMSF, NIAID
	R. H. Karstens	Bio Lab Tech (Micro)	LMSF, NIAID
	M. E. Schrupf	Bio Lab Tech (Micro)	LMSF, NIAID

COOPERATING UNITS (if any)

Rance LeFebvre (UC Davis); Will Probert (UC Davis); Alan Barbour (UTHSC); Patricia Rosa (LMSF); Mark Klempner (Tufts-NEMC); Warren Simpson (U. Otago, New Zealand)

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.2

PROFESSIONAL:

0.7

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Lyme disease is the most prevalent arthropod-borne disease of humans in the United States and many other countries throughout Europe and Asia. The objectives of this project are to (1) use recombinant DNA techniques to express specific antigens of *Borrelia burgdorferi* to improve the serodiagnosis of Lyme disease, (2) characterize at the molecular level, isolates of the Lyme disease spirochete from a wide range of biological and geographical sources, and (3) examine adaptive molecular responses produced by *B. burgdorferi* during infection in ticks.

Previously, we described the cloning and expression of a gene encoding a 39-kDa protein (P39) from *B. burgdorferi*, a causative agent of Lyme disease, which appeared to be an excellent candidate for a serological diagnostic antigen. Analysis of the completed DNA sequence revealed two adjacent open reading frames with a sequence identity of 62%. Gene 1, designated *bmpA* for borrelia membrane protein A, corresponds to a protein of 339 amino acids with a calculated molecular mass of 36.965 kDa and an estimated pI of 4.993. Because this gene encodes a recombinant protein that reacted by Western blot with all of 10 serum samples collected from human Lyme patients with previous erythema migrans or arthritis but not with 10 normal serum controls, it is assumed that this protein is equivalent to P39. The open reading frame of gene 2 has been designated *bmpB*. This open reading frame begins 116 nucleotides downstream of *bmpA* and potentially encodes a protein of 341 amino acids with a calculated molecular mass of 37.546 kDa, which we designate ORF2. The estimated pI is 4.859. A putative promoter 5' to the start codon of *bmpA* was present with *E. coli* consensus sigma-70 type -10 and -35 regions whereas *bmpB* lacked recognizable promoter elements. The deduced amino acid sequences of P39 and ORF2 were 52% identical. Both polypeptides had one or two charged amino acids after the initial methionine followed by several hydrophobic residues. At position 18 of P39 and position 15 of ORF2 there is a cysteine. This residue is preceded by the tripeptides FLS in P39 and LTS in ORF2. These sequences are consistent for a signal peptide with a signal peptidase II site at the cysteine. Both genes will be cloned separately into the pMAL expression vector, which produces a fusion product of the cloned gene and a maltose binding protein. Either fusion proteins or the purified proteins of *bmpA* and *bmpB* will then be tested for reactivity with anti-P39 antibodies. One or both of these purified recombinant proteins will then be made available for use as diagnostic antigens for Lyme disease serology tests.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00516-07 LMSF

PERIOD COVERED

October 1, 1993, to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunoglobulin Biology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	S. H. Pincus	Medical Officer	LMSF, NIAID
Others:	T. D. Duensing	IRTA Fellow	LMSF, NIAID
	H. Fang	Visiting Fellow	LMSF, NIAID
	V. Tolstikov	Visiting Scientist	LMSF, NIAID
	R. L. Cole	Chemist	LMSF, NIAID
	K. Messer	Bio Lab Tech	LMSF, NIAID
	R. Ireland	Microbiologist	LMSF, NIAID

COOPERATING UNITS (if any)

Chun Liu, Special Volunteer

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

6.0

PROFESSIONAL:

4.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The goal of this work is to study the structure and function of the immunoglobulin molecule. The work is being performed so that this understanding will be applied to the development of antibodies for human therapy.

A. Therapeutic systems. 1. Antibodies to surface antigens of group B streptococci (GBS) have been demonstrated to have protective efficacy in a model of neonatal sepsis. We have identified colony opacity variants of GBS, studied their interactions with host defenses, and shown that opacity differences are even more marked in clinical isolates than in the laboratory strains previously analyzed. 2. The efficacy of anti-HIV antibodies coupled to ricin A-chain has been studied *in vitro*. Antibodies directed against different envelope epitopes have been tested. Biological variants of HIV that escape killing with these immunotoxins and CD4-PE40 have been identified. The phenotype of the cells carrying these HIV has been studied, and the molecular mechanisms of immunotoxin escape have been evaluated. Immunotoxins are being tested *in vivo* in well-studied animal systems. 3. Evaluation of the anti-HIV antibody response in subjects exposed to the IIB/LAV isolate of HIV. Subjects include humans and chimpanzees infected with the virus or immunized with envelope subunits.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00549-06 LMSF

PERIOD COVERED

October 1, 1993, to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Mechanisms of Variation and Adaptation in *Borrelia burgdorferi*

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	P. A. Rosa	Senior Staff Fellow	LMSF, NIAID
Others:	K. L. Tilly	Senior Staff Fellow	LMSF, NIAID
	N. Margolis	IRTA Fellow	LMSF, NIAID
	B. Stevenson	IRTA Fellow	LMSF, NIAID
	D. M. Hogan	Microbiologist	LMSF, NIAID

COOPERATING UNITS (if any)

J. Fuhrman, Summer Student; J. Tekchandani, Summer Student; T. Schwan, LMSF; S. Samuels and J. Marconi, RMLMB; S. Barfield, Yale University

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.5

PROFESSIONAL:

2.75

OTHER:

0.75

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our broad objective is to understand the means by which *Borrelia burgdorferi* establishes an infection:transmission cycle between the tick vector and mammalian reservoir host, both of which are needed to maintain the spirochete in nature. The following research efforts relate to this goal.

1. Outer surface protein variation. The outer membrane of *B. burgdorferi* contains several abundant proteins (Osp) that vary in size and expression and are of unknown function. It is likely that the different Osps confer distinct properties on the spirochete that are pertinent to the different environments in which it must survive. Dr. Margolis cloned and sequenced an *ospC* gene that is variably expressed in culture and homologous to members of the large variable membrane protein family in *B. hermsii*. Analysis of the promoter region identified potential secondary structure in the RNA or DNA that could influence gene expression. Upstream of *ospC* are sequences homologous to 2 purine biosynthesis enzymes; the plasmid location of these *gua* genes is unique to *Borrelia* and may reflect an adaptation to disparate purine levels in ticks versus mammals. Dr. Tilly has demonstrated that one of these genes encodes a functional product by complementing an *E. coli* mutant deficient in this enzyme. Overlapping divergent promoters could permit co-regulated expression of the *ospC* and *gua* genes.

2. Plasmid structure and replication. Understanding the structure and replication of the unusual linear and circular *Borrelia* genome is of intrinsic interest and practical merit. Dr. Tilly has assayed *Borrelia* extracts for proteins that bind to telomeric sequences. She has isolated and sequenced the gene for the *Borrelia* HU homolog, a gene that may be involved in linear plasmid structure and replication. She has determined the genetic organization of the region, which includes two unknown open reading frames and homologs of the *rho* and *rpst* genes and complemented *E. coli* mutants defective in some homologous genes. Hogan and Rosa have used a library constructed by Fuhrman to walk upstream from the *ospAB* operon toward the telomere of the 49-kb linear plasmid, which is predicted to have different sequence from those previously cloned. Hogan has identified evidence for an unusual integration event of sequences from one circular plasmid into another.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00568-05 LMSF

PERIOD COVERED

October 1, 1993, to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Basis and Extent of Microbial Sex

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	J. A. Heinemann	Staff Fellow	LMSF, NIAID
Others:	R. G. Ankenbauer	IRTA	LMSF, NIAID
	J. D. Klena	IRTA	LMSF, NIAID
	T. D. Duensing	IRTA	LMSF, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1

PROFESSIONAL:

1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

My laboratory studies two examples of accelerated genetic changes in microbes. The first is called bacterial conjugation, a process that permits the exchange of genetic information between cells and has the potential to deliver as much as an entire bacterial chromosome. Whereas traits are normally inherited according to Mendelian principles, through passage from parents to offspring, bacteria can exchange genes with neighbors thereby creating hybrid genotypes. Conjugation is mediated by plasmids that can be transferred from many bacterial species to a large range of heterologous microbes, including eukaryotes. The second, called directed or Cairnsian mutation, may engage a quasi-Lamarckian strategy to accelerate genetic changes when microbes encounter a lethal challenge. Although the molecular details of this mechanism remain to be elucidated, documentation of its existence in bacteria and yeast is accumulating. My laboratory has explored a putative example of Cairnsian adaptation in eukaryotes. We found that the phenomenon was an unexpected and partial adaptation to environmental challenge that could be an intermediate phenotype in the pathway to full adaptation.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00608-04 LMSF

PERIOD COVERED

October 1, 1993, to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Expression and Phase Variation of Gonococci opa Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	R. J. Belland	Visiting Associate	LMSF, NIAID
Others:	T. Chen	Visiting Fellow	LMSF, NIAID
	J. Swanson	Chief	LMSF, NIAID
	J. Carlson	IRTA	LMSF, NIAID
	S. Morrison	Microbiologist	LMSF, NIAID
	D. Hogan	Microbiologist	LMSF, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

2.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

*Neisseria gonorrhoeae*, the causative agent of gonorrhea, expresses one or more members of the opa multigene family during the course of disease. We have studied the expression mechanisms and the biological properties of the expressed outer-membrane proteins to more clearly understand the role of this protein family in the pathogenesis of gonorrheal disease.

Phase variation (on/off expression changes) occurs through a translational frameshifting mechanism dependent on "non-homologous" recombination in a series of direct, tandem repeats within each opa gene. This mechanism has been used as a paradigm for a number of other neisserial genes which phase vary using similar repetitive stretches of DNA. We have determined a number of cellular functions, primarily DNA replication and repair enzymes, which significantly influence the rates at which opa genes (and others using similar mechanisms) phase vary. Attempts to mutate these genes in *N. gonorrhoeae* are underway with the primary goal of creating bacterial strains which can no longer regulate phase variation of these genes at normal levels.

Expression of outer-membrane Opa proteins influence the adhesive properties of *N. gonorrhoeae* towards epithelial cells and PMNs. We have studied the nature of the adherence and have shown that certain Opa proteins bind a family of compounds termed glycosaminoglycans, found on the surface of numerous cell types and in the external mucosal environment. We have also shown that the expression of Opa proteins may protect Opa+ bacteria from the bactericidal components of human serum in the presence of human urine. This effect is similar to that seen with added heparin and studies are underway to further characterize the nature of the protective components in human urine.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 AI 00609-03 LMSF

PERIOD COVERED

October 1, 1993, to November 7, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Synthetic Gene Approach to Study the Action of Antimicrobial Genes of Mammals

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: K. S. Bhat

Senior Staff Fellow

LMSF, NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects   ☐ (b) Human tissues   ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project terminated on the departure of Dr. Bhat.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
ZO1 AI 00632-03 LMSF

PERIOD COVERED

October 1, 1993, to April 30, 1994

TITLE OF PROJECT (90 characters or less. Title must fit on one line between the borders.)

Siderophores and Iron Transport in *Pseudomonas aeruginosa*

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: R. G. Ankenbauer IRTA Fellow LMSF, NIAID

Other: C. A. Dooley Pre-IRTA Fellow LMSF, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.2

PROFESSIONAL:

0.6

OTHER:

0.6

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

*Pseudomonas aeruginosa* is an important opportunistic pathogen responsible for high mortality in cystic fibrosis and hospital-acquired infections. The damage caused by *Pseudomonas aeruginosa* is highly dependent upon the ability of the organism to obtain iron from the host. If iron is not available to the organism, the bacterium is unable to grow or cause disease. I am concentrating on the study of how this bacterium acquires iron from the host. *P. aeruginosa* produces iron-sequestering compounds called siderophores which help the bacterium get iron from the host or environment. One of these siderophores is called pyochelin and is the subject of my work. The bacterium is able to transport iron into the cell when it is bound to pyochelin. This occurs through a surface protein receptor on the bacterium. The gene for this surface protein has been cloned and sequenced. The receptor for the iron-pyochelin complex is quite similar to receptors for other iron-siderophore complexes which have little structural similarities to the iron-pyochelin complex. This result indicates that many bacteria use very similar systems for the transport of iron into the cell. The similarities of these systems suggest that anti-microbial chemotherapy based on iron-siderophore transport systems may be applicable to a broad range of bacterial infections. An additional finding is that the iron-pyochelin receptor is expressed only under conditions of low iron available. This iron-regulated production of the receptor is caused by a specific DNA sequence called the 'iron box', and this is the first example of such a DNA sequence to be identified in *P. aeruginosa*.

This project terminated April 30, 1994, on the departure of Dr. Ankenbauer



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00633-02 LMSF

PERIOD COVERED

October 1, 1993, to March 16, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Neisserial-Host Interaction: Pathogenic Mechanisms and Host Responses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. H. Fischer

Research Associate

LMSF, NIAID

COOPERATING UNITS (if any)

M. Konkel, LVP, RML, NIAID

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.4

PROFESSIONAL:

0.4

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects   ☐ (b) Human tissues   ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project terminated on the departure of Dr. Fischer.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00694-01 LMSF

PERIOD COVERED

April 3, 1994, to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic Analysis of the Gonococcus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: S. A. Hill Staff Fellow LMSF, NIAID

Others: J. Swanson Chief LMSF, NIAID

R. J. Belland Visiting Associate LMSF, NIAID

COOPERATING UNITS (if any)

Frank Stahl, University of Oregon; Rosemary Redfield, University of British Columbia

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

My broad objective is to understand how recombination impacts upon the biology of *Neisseria gonorrhoeae* with special emphasis towards elucidating the molecular mechanism underlying pilin antigenic variation. The *neisseria* as a genus show a remarkable propensity for undergoing homologous recombination both within a species and between closely related members of the genus. Such promiscuity allows the dissemination of chromosomal genetic elements throughout a population within nature, leading to a large repertoire of variant subtypes. Consequently, the presence of large variant populations makes therapeutic intervention more difficult and impairs the efficacy of many vaccine candidates.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1993 to September 30, 1994

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Host and Viral Factors in Resistance to Rabies Virus Infection in Mice

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. L. Lodmell

Scientist Director

LPVD, NIAID

## Other:

Nancy B. Ray, Ph.D.

IRTA Fellow

LPVD, NIAID

## COOPERATING UNITS (if any)

Jean Smith, M.S.

CDCP

## LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT 59840

## SECTION

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL:

2.0

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The principal objectives of this research project are five-fold: 1) to determine host and viral factors which influence the genetically controlled resistance of inbred mouse strains to street rabies virus (SRV) 2) to determine if an *in vivo* laboratory model for persistent rabies virus infection can be established with subsequent goals of determining in which cell(s) the virus (genome) is sequestered and whether the virus can be activated to produce clinical disease and death 3) to understand the role of macrophages in rabies virus infections 4) to compare the protective capabilities of recombinant vaccines expressing different or multiple structural proteins of the rabies virus against genetic variants of rabies virus that have been isolated worldwide and 5) to advance our understanding of the complex interaction between microglial cells, lymphocytes and cytokines in the pathogenesis of rabies virus infections. Studies have determined that anti-rabies virus nucleoprotein antisera is protective and inhibits rabies virus replication. Experiments using a recombinant vaccine dually expressing this highly conserved protein and the glycoprotein of the rabies virus against genetic variants of rabies virus isolated worldwide are in progress. Persistent rabies virus infections have been established in several strains of inbred mice. Attempts to isolate infectious virus with an *in vitro* amplification technique from tissues of these mice have been discouraging. Nonetheless, virus was isolated from either bone marrow or central nervous system tissue of 5/47 (11%) mice which had been infected up to 90 days previously. Experiments to enhance the sensitivity of our detection assay have determined that our *in vitro* amplification assay is >100 fold more sensitive than detection of mRNA by Northern blot analysis. To increase sensitivity, we are now using RT-PCR. Preliminary results are encouraging in that the ERA rabies virus has been amplified and detected in tissue culture infected cells with both nucleoprotein and glycoprotein primers. Unfortunately, amplification and detection *in vitro* of the bat street rabies virus which was principally used to establish persistent infections in mice have failed. Presently we are testing nested PCR reactions and new sets of nucleoprotein and glycoprotein primers to detect this virus. Further progress on this project depends on our ability to initially detect *in vitro* the viruses which were used to establish the *in vivo* persistent infections. We have determined that both tissue culture adapted and non-tissue culture adapted wild strains of rabies viruses replicate in primary murine macrophages and murine and human macrophage-like cell lines. Persistent infections were established in U937 cells with 2 different strains of virus. Rabies virus replicated in differentiated, but not undifferentiated, HL-60 cells. This data in conjunction with that of the macrophage-like cell lines suggests that more differentiated cells are more permissive to rabies infection. Studies to determine whether microglial cells serve as reservoirs of infection in persistent infections and also contribute to rabies-induced pathology of the brain are in progress.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 AI 00074-22 LPVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetically Controlled Mechanisms of Pathogenesis and Recovery in Friend Retrovirus-Induced Leukemia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. Chesebro Chief LPVD, NIAID

Other: K. Hasenkrug IRTA LPVD, NIAID

COOPERATING UNITS (if any)

M. Miyazawa, Department of Pathology, Tohoku University, Sendai, Japan; L. Perry, Department of Veterinary Microbiology and Pathology, School of Veterinary Medicine, Washington State University, Pullman, WA

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

1.2

OTHER:

2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews This is a non-clinical AIDS-related project.

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Friend virus (FV) is a complex of 2 mouse retroviruses which induce rapid erythroleukemia in many strains of adult mice. Various genes in susceptible mice can modify the outcome of FV infection. Our laboratory has identified 5 such genes including 4 major histocompatibility complex (MHC) regions (D, IA, IE and T) and one non-MHC gene, Rfv-3. These genes all influence the host immune response to FV and FV leukemia cells and can even induce spontaneous recovery from leukemia or facilitate successful protective vaccination against challenge with live FV or FV leukemia cells.

This years work studied the mechanism of the influence of the MHC D region on recovery. Using mice expressing only one allele of the  $\beta_2$ -microglobulin gene caused a 50% reduction in the expression of the MHC D region gene product. However this did not reduce the incidence of recovery from FV leukemia indicating that heterozygous levels of the D<sup>b</sup> allele are adequate for recovery. The results suggest that the non-recovery D<sup>d</sup> allele or the closely linked L<sup>d</sup> allele might cause a negative influence on the recovery process.

The MHC IE region was also studied in this system, and it was found to exert both a positive and a negative influence on recovery from leukemia. The positive affect was due to the role of the E molecule on presentation of FV envelope protein antigens to the immune system. The negative effect appeared to act by its influence on selection of T cell subsets during ontogeny.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 AI 00085-17 LPVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pathogenesis of Aleutian Disease Virus Infection

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. E. Bloom

Medical Officer

LPVD, NIAID

Other: Linda Dworak

Staff Fellow

LPVD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.9

PROFESSIONAL:

1.0

OTHER:

0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

This is a non-clinical IIDEA project.

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Aleutian mink disease parvovirus (ADV) causes a persistent infection that is restricted in adult animals and is associated with disturbances of immune regulation. Features of Aleutian disease (AD) include polyclonal hypergammaglobulinemia, plasmacytosis, immune complex disease, interstitial and glomerulonephritis and exceedingly high levels of anti-ADV antibodies. The spectrum of findings resembles those associated with a Th2 pattern of cytokine responses. The scope of this project is to elucidate mechanisms by which ADV infection results in this unusual disorder.

We found that levels of the cytokine Interleukin-6 (IL-6) increased as mink infected with ADV develop disease. Furthermore, amounts of IL-6 mRNA also appeared to increase as mink developed AD. Conditions in which IL-6 is overexpressed in human and mice resemble the clinicopathologic picture of ADV infection in adult mink. Inappropriate production of IL-6, and possibly other cytokines, during chronic ADV infection may play a role in the generation of the immune disorders. This would be consistent with a Th2 type of immune response.

Analysis of transcription suggested that restricted ADV infection may be associated with reduced synthesis of either capsid proteins or nonstructural protein 2 (NS2). Disrupted NS2 synthesis has been found to cause nonpermissive infection for other parvoviruses in a cell type specific fashion.

Infected mink do not generate a detectable antibody response against all regions of the various nonstructural (NS) proteins. When sera were tested against fusion proteins containing open reading frames specific for segments of the NS proteins, we found antibodies directed against NS1 specific regions and the amino-terminal region common to all NS proteins, but no reactivity against the NS2 specific ORF. An antibody prepared against the NS2 specific ORF detected a protein of the correct size indicating that NS2 is a bona fide gene product in permissively infected cells. Consequently, NS2 may not be expressed in vivo during ADV infection. This finding might implicate disordered NS2 synthesis in restricted in vivo infection.







## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1993 to September 30, 1994

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pathogenesis of Diseases Induced by Non-Oncogenic Retroviruses

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. L. Portis Medical Officer LPVD, NIAID

Other: William Lynch Staff Fellow LPVD, NIAID

Richard Bessen IRTA Fellow LPVD, NIAID

## COOPERATING UNITS (if any)

Transgenic Mouse Facility, NIAID, Frederick MD

## LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT 59840

## SECTION

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.1

## PROFESSIONAL:

3.0

## OTHER:

1.1

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

This is a non-clinical AIDS-related project.

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The retroviruses we study were isolated from wild mice and cause a non-inflammatory neurodegenerative disease in mice similar to that caused by the unconventional agents such as scrapie. We are using this animal model to study the pathogenesis of retrovirus-induced neurodegeneration.

**Implant studies:** The mechanism of neurodegeneration appears to be indirect since the neurons which degenerate appear not to be infected. We showed that microglial cells are a important target of virus infection and that infected microglial cells colocalized with the neuronal cytopathology. We have now shown that microglial cells isolated from the brains of neonatal mice, infected *in vitro* and implanted into the brains of 10 day old can induce local spongiform neuropathology. The cells migrate to specific sites within the brain remote from the sites of injection and lesions have been detected in the thalamus, colliculus and along the corpus callosum. This is the first direct demonstration that microglial cells infected with a retrovirus may be neurotoxic *in vivo*, a finding which has direct implications to understanding HIV encephalopathy.

**Envelope protein:** The viral envelope protein contains the determinants of neurovirulence, but the nature of this effect is not understood. We used two related retroviruses, both of which infect the brain at high levels, but only one of which is neurovirulent. The viral envelope protein was examined in microglial cells infected *in vitro* with each virus. Interestingly, microglial cells infected with the neurovirulent virus expressed only the precursor of the envelope protein, suggesting that proteolytic cleavage of this protein did not occur in these cells. In contrast, the *env* precursor was proteolytically cleaved in cells infected with the non-neurovirulent virus. Since both of these viruses infect a comparable spectrum of cell types in the brain, this suggests that proteolytic processing may be proximally involved in disease pathogenesis.

***In vitro* model of neurodegeneration:** We are continuing our attempts to develop an organotypic slice culture system which will recapitulate the neuropathology seen *in vivo*. Infected slice cultures derived from the brain stem and the spinal cord have been maintained for weeks in culture. The retrovirus infection is persistent and the infected cells have been identified by dual color immunofluorescence using cell-type specific markers. The major infected cell in spinal cord cultures is the microglial cell. The viral protein profile expressed in these cultures has also been followed as a function of time after infection. As seen *in vivo* there are several isoforms of envelope protein whereas the precursor of the core (*gag*) proteins is expressed as a single isoform. Thus, both the target cells of the infection *in vitro* and the processing of the viral proteins appears to mimic that seen in mice inoculated with this virus.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 AI 00262-13 LPVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Pentraxins in Hamster Physiology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. E. Coe Medical Officer LPVD, NIAID

Other:

COOPERATING UNITS (if any)

Drs. B. Canquillhem & P. Pevet, Strasbourg, France; Dr. B. Dowton, Wash. Univ. Med. School, St. Louis, MO; Dr. K. Ishak, AFIP, Washington, D.C.; Dr. U. Nilsson, Uppsala, Sweden; Dr. Mortensen, OSU, Columbus, OH; Dr. D. Johnson, U. Kansas Med. Center, Kansas City; Dr. Gary Neisestuen, U. Minn., St. Paul; Dr. J. Ward, NCI, Ft. Dietrick, MD; Dr. T. Duclos, U. New Mex., Albuquerque, NM; Dr. H. Gewurtz, Rush Med. Center, Chicago, IL

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT 59840

SECTION

NIAID, NIH, Bethesda, MD 20892

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.75

PROFESSIONAL:

.75

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Syrian hamster has an unusual serum protein under control of sex hormones. This protein is called female protein (FP) because of its special expression in females where serum levels are 100-200 fold greater than in normal males. FP is a homolog of two human proteins: C reactive protein (CRP) and serum amyloid P component (SAP). This family of proteins is called pentraxin and are found in most animals and birds. Pentraxins have evolved with little change in structure. These findings would imply an important function of pentraxins, however, a raison d'être has not been described. FP shares function-properties with both human pentraxins such as Ca<sup>++</sup> dependent phosphorylcholine binding, complement fixation, acute phase responsiveness (characteristics of CRP) and also is a constituent of amyloid (characteristic of SAP). Indeed, high serum levels of FP occurring naturally (as in female) or experimentally (as in hormonally treated male) are directly associated with deposition of amyloid in Syrian hamsters. The high serum levels of FP may be a primary cause of hamster amyloidosis. Thus, females treated with testosterone to lower serum FP levels do not acquire amyloidosis as early in life as do normal hamster females. Estrogen administered to male hamsters enhances expression of amyloidosis; however, only those estrogens which increase FP synthesis (such as diethylstilbestrol) will have this effect. The sex hormone control of FP synthesis in the Syrian hamster provides a unique opportunity to examine the role of this pentraxin in amyloidosis. FP synthesis is under different control mechanism in other hamsters. For example, in Armenian hamster, FP is down regulated by estrogen administration, and this turn off of FP synthesis may be related to the acute hepatic toxicity and chronic hepatocarcinogenesis induced by exogenous estrogens. A variety of plant foods contain estrogens (mycoestrogens and phytoestrogens), and these foods may be responsible for the known influence of diet on development of certain human diseases such as breast and prostate cancer. Because hamsters are so sensitive to the effects of estrogens, we have been feeding hamsters various estrogenic foods to determine if dietary estrogens can 1) alter hepatic synthesis of FP, 2) change expression of amyloidosis, 3) influence the estrogen initiation of kidney and liver tumors. Because of technical problems, these experimental results are preliminary but do indicate that flax consumption may alter expression of amyloidosis, and a soy bean diet may change hepatic FP synthesis and influence the hepatic response to exogenous estrogens.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1993 to September 30, 1994

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and Function of the ADV Genome

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. E. Bloom

Medical Officer

LPVD, NIAID

Other: Torben Storgaard

Special Volunteer

LPVD, NIAID

## COOPERATING UNITS (if any)

Department of Veterinary Virology, Royal Danish Veterinary University, Copenhagen, Denmark

## LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT 59840

## SECTION

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.9

## PROFESSIONAL:

1.0

## OTHER:

0.9

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

This is a non-clinical IIDEA project.

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The ongoing purpose of this project is to compare the genome structure of pathogenic and nonpathogenic strains of Aleutian mink disease parvovirus (ADV) and to relate these differences to the ability of the various isolates of virus to replicate in cell culture and to cause disease in infected mink.

By constructing clones that are chimeric between the nonpathogenic ADV-G (type 1 ADV) and ADV-Utah 1 (type 2 ADV), we have now identified a segment that influences the pathogenicity of ADV. Molecular clones of ADV-G that contained the 73-88 map unit piece of ADV-Utah 2 replicated 2log10 less efficiently than the ADV-G parent in cell culture, but induced antibody and caused disease in mink injected with virus derived from these clones. This segment maps to the capsid protein coding sequences and there are 4 coding differences between the type 1 and type 2 virus. Furthermore, the region is distinct from the 54-63 map unit capsid gene segment that governs permissivity in cell culture. However, computer analysis and comparison with the sequence of canine parvovirus (CPV) suggests that both regions align with ones on the CPV capsid regulating host range and pathogenicity.

In order to relate these findings to the ADV virion structure, we have begun work to crystallize ADV. We are utilizing a recombinant baculovirus that synthesizes capsid proteins. The capsid proteins self-assemble into particles with physical characteristics of empty ADV virions.

In other work, we have begun studies to analyze structure-function interactions of the ADV P36 promoter. The sequence and spacing of ADV P36 elements differs significantly from other prototypic parvoviruses and these variations may explain the low constitutive and trans-activated strength of the promoter. A number of alterations were produced in the promoter, some of which "restored" promoter strength in CAT assays, but when the changes were introduced into full-length clones of ADV, all the clones were replication defective.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 A1 00265-13 LPVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunobiology of Scrapie Virus Infection

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. E. Race Research Veterinarian LPVD, NIAID

Other: B. Caughey Staff Fellow LPVD, NIAID

S. Priola Staff Fellow LPVD, NIAID

B. Chesebro Chief LPVD, NIAID

COOPERATING UNITS (If any)

Dr. A. Haase, Chief, Dept. Microbiology, University of Minnesota, Minneapolis, MN; Dr. Al Jenny, U.S.D.A., Ames, IA; Dr. Lyle Miller, Iowa State University; Dr. Randall Cutlip, U.S.D.A.; Dr. Michael Oldstone, Scripps Clinic, San Diego CA; Dr. Jean Manson, Edinburgh.

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.9

PROFESSIONAL:

1.9

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Scrapie is a spongiform encephalopathy of sheep and goats which can be transmitted experimentally to several other animal species. Similar diseases are recognized in cattle and humans. No etiologic agent has been identified. However, the proteinase K resistant form (PrP-res) of an endogenous protein designated prion protein (PrP) purifies with infectivity and is important to disease pathogenesis.

We developed a sensitive assay for PrP-res and utilized it to diagnose scrapie in sheep. Analysis based on PrP-res detection was much more accurate and less subjective than the currently used method of diagnosis based on the microscopic evaluation of brain. We also showed that PrP-res analysis of spleen or lymph node was nearly as accurate as analysis of brain. We have also shown that PrP-res accumulates prior to clinical disease in sheep lymph node and placenta. Thus analysis of sheep placenta or lymph node provides an ante mortem test for infection. We are also using PrP-res analyses to test tissues from cattle in order to determine if spongiform encephalopathy currently exists in U.S. cattle and, thereby, whether an epidemic similar to BSE in Great Britain is possible in the U.S.A. PrP-res analysis should also be relevant for diagnosis of the human disease counterparts.

The influence of specific PrP gene sequences on interspecies transmission of spongiform encephalopathies is also being studied. To do so, we have expressed various mouse-hamster PrP constructs in scrapie-infected mouse neuroblastoma (MNB) cells and are now analyzing them in mice and hamsters to determine if species tropism has been altered. Mouse neuroblastoma cells are also being used to study the normal function of the PrP protein as well as to identify factors which might account for the biochemical changes which lead to the conversion of the endogenous PrP protein to the disease associated PrP-res form.

Similar experiments are being done in vivo using transgenic mice containing the hamster PrP gene.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 AI 00266-13 LPVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic Structure of Murine Retroviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. H. Evans Chemist LPVD, NIAID

Other: M. Lavignon Fogarty Fellow LPVD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The major focus of research in this laboratory is the genetic variation of retroviruses, including variation as a result of point mutation as well as variation due to recombination of retroviral genes. Genetic variation occurs in all retrovirus infections, including avian, murine and human retroviruses such as HIV. Most of our current studies focus on the polymorphism of recombinant host range retrovirus variants and the characterization of endogenous retroviral sequences which are involved in the generation of the variants. Inoculation of many murine retroviruses in mice results in the generation of host range variants by recombination of the inoculated virus with endogenous retroviral genes. We have identified two major antigenic subgroups of these variants distinguished by their reactivity with two monoclonal antibodies (mAbs) and have defined the position of a single amino-acid residue whose identity determines reactivity to the antibodies. Our results indicate that the two subgroups are the result of recombination of inoculated virus with two distinct families of endogenous retroviral sequences. Furthermore, we have found that inoculation of different retroviruses results in the generation of strikingly different proportions of the variant subgroups suggesting that different inoculated retroviruses preferentially recombine with different populations of endogenous retroviral genes. We are currently comparing the endogenously-derived gene sequences of variant viruses with each other and with retrovirus gene sequences found in uninfected mice. These studies should precisely identify which endogenous sequences participate in recombination to generate the host range variants.

A second aspect of our studies is the mutation rate of retroviruses. In previous studies we have directly determined the mutation rate of a murine retrovirus and found that most progeny retroviruses do not sustain any point mutations during a single cycle in a fibroblastic cell line. We wish to extend these studies to determine if mutation rates are accelerated in different cell types, such as macrophages, and examine conditions which may alter mutation rates. Direct determinations of mutation rates are quite labor intensive and would limit progress in these studies. We are developing an alternative approach which exploits an antigenic conversion we have been able to effect by a single point mutation.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 AI 00418-11 LPVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular biology of Equine Infectious Anemia Virus, a Retrovirus Model for AIDS

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W. Maury Staff Fellow LPVD, NIAID

Other: B. Chesebro Chief LPVD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.4

PROFESSIONAL:

1.1

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews This is a non-clinical AIDS-related project.

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The horse lentivirus, equine infectious anemia virus (EIAV), is a retrovirus closely related to the human immunodeficiency virus. EIAV is useful as a model for the study of retroviral expression and latency.

A. The influence of the virus-encoded, trans-activating protein, Tat, on EIAV LTR activity continues to be investigated. We have shown that EIAV Tat is able to upregulate EIAV gene expression in a number of some, but not all cell lines. Additionally, not all cell lines which support EIAV transactivation support HIV transactivation. Regions within the EIAV LTR and Tat peptide which are important in transactivation have been identified. In cells highly permissive for EIAV expression, the EIAV R sequences which contain the Tat binding site, TAR, were the only EIAV specific sequences required; HIV enhancer/promoter sequences were able to substitute for analogous sequences within EIAV despite the virtual absence of sequence homology. In contrast, both EIAV and HIV enhancer sequences were required for maximal transactivation in cells restricted for EIAV expression. This work has been published and no further work on this project in ongoing at this time. As an extension of these studies, mapping of LTR sequences required for basal and transactivated levels of expression in tissue culture cells and primary horse macrophages are ongoing. We recently published work in which EIAV enhancer sequences important for virus expression in primary macrophages were characterized. A protein that interacts with these enhancer sequences and appears to be regulate EIAV expression was identified. In addition, the enhancer sequences and the proteins which bind to those sequences in non-permissive and permissive tissue culture cell lines is being explored. At the present time, these studies involve electrophoretic gel shifting assays as well as transient transfection assays for detection of viral gene expression.

B. Latency and viral reactivation in an field infected, EIAV-seropositive mare is being investigated. This mare has never been clinically ill with EIAV. No infectious virus has ever been isolated from her, however, by immunocompromising the mare EIAV sequences have been detected by PCR in her peripheral blood monocytes. Further characterization of the latent state of EIAV was tested by passage of whole blood from this mare into a naive animal. This animal seroconverted, but did not become acutely ill. The field-infected mare has now been donated to Washington State University for an ongoing collaborative effort to obtain virus and/or viral sequence data from this latently infected horse. Such studies should provide both viral sequence information as well as information on virus location within a latently infected host.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 AI 00468-09 LPVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biology of Human AIDS Retrovirus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. Chesebro Chief LPVD, NIAID

Other: W. Maury Staff Fellow LPVD, NIAID

K. Toohy IRTA Fellow LPVD, NIAID

COOPERATING UNITS (if any)

Diane Griffin, M.D., Department of Neurology, Johns Hopkins University School of Medicine; Richard Johnson, M.D., Department of Neurology, Johns Hopkins University School of Medicine; Chris Power, M.D., Department of Neurology, Johns Hopkins University School of Medicine; David Kabat, Ph.D., Department of Biochemistry, Oregon Health Sciences Center, Portland, OR.

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.9

PROFESSIONAL:

2.4

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

This is a non-clinical AIDS-related project.

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Human immunodeficiency virus (HIV) infection is associated with several different clinical syndromes including immunosuppression, Kaposi's sarcoma, wasting syndrome, and neurological abnormalities such as dementia. The goal of this project is to understand the role of variation in the viral genes in causing these diverse syndromes.

We recently showed that HIV strains with ability to infect macrophages shared a strong homology in the amino acid sequences of their envelope proteins in the hypervariable V3 region. Other work has now confirmed that such macrophage-tropic HIV strains appear to be the main ones sexually transmitted between different individuals. The wide diversity of V3 sequences seen at later disease stages appears to evolve independently in each infected individual. Thus, the macrophage-tropic envelope consensus sequence may be an ideal target to which potential vaccines should be directed.

In more recent studies we have analyzed V3 envelope sequences from a group of AIDS patients with and without HIV dementia. Whereas brain derived HIV from both groups appeared to be closely related to macrophage-tropic HIV strains, viruses from demented patients showed significant differences from those from non-demented patients. These results suggested that HIV dementia might be caused by a unique subset of macrophage-tropic HIV strains.

The ability of primary AIDS patient HIV isolates and laboratory-adapted HIV isolates to infect cells *in vitro* was studied using HeLa-CD4 clones expressing differing amounts of CD4. Laboratory-adapted HIV showed a similar level of infectivity over a wide range of CD4 expression. In contrast, infectivity of primary patient HIV isolates was directly proportional to the amount of CD4 expressed on HeLa cells. These results demonstrate important functional differences in the envelope genes of primary patient HIV isolates which could be involved in various *in vivo* pathogenic effects.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 AI 00580-05 LPVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biochemistry of Scrapie Pathogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI: B. Caughey, Ph.D. Senior Staff Fellow LPVD

Other: Richard Race, D.V.M. Research Veterinarian LPVD

Bruce Chesebro, M.D. Chief LPVD

Gil Katzenstein, Ph.D. IRTA Fellow LPVD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.3

PROFESSIONAL:

2.3

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

This is a non-clinical IIDEA project.

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The infectious agent of scrapie and other transmissible spongiform encephalopathies (TSE) resembles a virus in that it replicates *in vivo* and has distinct strains. Nevertheless, the agent has been proposed to contain only PrP-res, a neuropathogenic, protease-resistant form of the host-encoded prion protein. We have shown previously that PrP-res is derived post-translationally from normal, protease-sensitive PrP (PrP-sen) in scrapie-infected cells. However, the conversion mechanism and the relationship of PrP-res formation to TSE agent replication remain unclear, in part because the conversion was never accomplished in a defined, cell-free system. We have now converted PrP-sen to protease-resistant forms similar to scrapie associated PrP-res in a cell-free system composed of substantially purified constituents. This conversion required the presence of preexisting PrP-res, providing the first direct evidence that PrP-res derives from interactions between PrP-sen and PrP-res. Denaturation of the preexisting PrP-res prevented conversion, indicating that maintenance of some native structure of PrP-res was required. Our present studies with this reconstitution system are aimed at defining the conversion mechanism and specificity of the conversion as it relates to PrP genotype and scrapie strain. With further refinements of this system, a clear evaluation of how protease-resistant PrP relates to TSE infectivity may finally be possible.

Another approach we have taken to defining the mechanism of PrP-res formation and how it might be blocked for therapeutic purposes is to identify and characterize inhibitors of PrP-res accumulation scrapie-infected mouse neuroblastoma cells. Having already shown that Congo red and certain sulfated glycans are potent inhibitors, we proceeded to compare the potencies of numerous additional sulfated and sulfonated polymers and Congo red analogs in order to better define the attributes of such inhibitors. These studies have shown that there is great molecular specificity in the inhibition mechanism because even subtle changes in inhibitor structure, such as moving a pair of methyl groups on the biphenyl group of Congo red analogs, can cause dramatic differences in potency. Such specificity is important in potential pharmacological applications of such inhibitors.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 AI 00611-04 LPVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Enrichment of Hematopoietic Stem Cells from Mouse Bone Marrow

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: G.J. Spangrude, Ph.D. NIAID Investigator LPVD, NIAID

Other: D.B. Tumas, D.V.M., Ph.D. Staff Fellow LPVD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Hematopoiesis is an ongoing developmental process in adult mammals. The details of the early stages of this process remain obscure, mainly because the very low frequency of hematopoietic stem cells (HSC) in bone marrow limits the ability to study these cells. The goal of this project is to define phenotypic characteristics which will help to identify HSC as they normally exist in bone marrow tissue. Further, this project aims to document growth and differentiation characteristics of normal HSC by transplantation studies and by in vitro culture techniques. We are also interested in defining optimal conditions for infecting enriched populations of HSC with retrovirus constructs. We have developed an enrichment scheme that reproducibly recovers a full spectrum of hematopoietic progenitors from mouse bone marrow. Within this population we can identify a subset of cells that can repopulate irradiated animals at near-single cell levels. We have performed transplantation studies using very small numbers of purified HSC to demonstrate that the cell population we have identified can contribute to hematopoiesis over the life-span of the recipient animals. Further, we have followed the progeny of those cells as they contribute to the peripheral blood and have shown two general patterns of repopulation, one in which significant repopulation is seen within 5 weeks of the transplant, and another in which little repopulation is seen until later times. In both cases, contribution to the peripheral blood is maintained over long periods of time. It is unclear whether the two types of long-term repopulation are due to two distinct stem cell types, or rather are due to differential seeding to specific microenvironments in the host animal. These experiments demonstrate the potential of individual HSC to function over extended periods of time. We have obtained quantitative information on the ability of HSC to self-renew. This has been approached by re-isolating HSC progeny from transplanted animals many months after the original transplant and testing their repopulating potential. Although we have seen expansion of cells expressing the HSC antigen phenotype, we do not see a parallel expansion of HSC function. This demonstrates that phenotype alone can not be used to indicate HSC function. We have also investigated expression of the multi-drug resistance phenotype in these cells, their cell cycle status, and the ability of Friend virus-derived vectors to introduce and express foreign genes in the hematopoietic system.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 AI 00673-02 LPVD

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Estrogen Hepatotoxicity and Hepatocarcinogenicity in Hamsters

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. E. Coe Medical Officer LPVD, NIAID

Other: M. Satoh Visiting Fellow LPVD, NIAID

COOPERATING UNITS (if any)

Dr. K. Ishak, AFIP, Washington, D.C.; Dr. J. Ward, NCI, Ft. Dietrick, MD

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT 59840

SECTION

NIAID, NIH, Bethesda, MD 20892

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.25

PROFESSIONAL:

1.25

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided..)

Estrogens are known to be etiologic in human breast cancer and also in human liver tumors. Estrogen can also adversely affect hepatic function in certain genetically susceptible women where it causes a disease called cholestatic jaundice of pregnancy. We have found that the liver of Armenian hamster is extraordinarily sensitive to the effects of exogenous estrogens. Therefore, this rodent can be used as a new experimental model to study how estrogen disrupts hepatobiliary function to cause icterus and how estrogen produces hepatic tumors. We have used primary tissue culture of liver cells from the Armenian hamster in an attempt to reproduce *in vitro* the pathophysiology observed *in vivo* after estrogen administration. As a control, we have used primary hepatic cultures obtained from Syrian hamsters because in this hamster administration of estrogen does not produce acute hepatic toxicity or chronic hepatocarcinogenesis. In general, we have not been able to reproduce *in vitro* the hepatocyte toxicity from estrogens that has been observed *in vivo*. A cytoplasmic inclusion body (related to the human hepatic inclusion body called Mallory body) has been observed to appear after treatment of primary cultures of Armenian hamster hepatocytes. Also, an aberrant response has been found in primary hepatocyte cultures obtained from Armenian hamsters previously treated with estrogen. We do not know what unknown factors are necessary *in vitro* to reproduce the known estrogen toxicity observed *in vivo*.



ROCKY MOUNTAIN LABORATORIES MICROSCOPY BRANCH  
 Rocky Mountain Laboratories  
 Hamilton, Montana  
 1994 Annual Report  
 Table of Contents

Z01 AI		
Project Number		Page
Summary		21- 1
00488-08	Ultrastructural Analysis of Antigenic Determinants In Pathogens - Garon	21-9
00554-06	Structural Characterization of Microbial Genes and Nucleic Acid Molecules - Garon	21-10
00670-02	The Genetic and Physiological Basis of Acid Resistance in Enteric Bacteria - Small	21-11
00671-02	The Molecular and Cell Biology of Pathogenic Mycobacteria Species - Small	21-12



ANNUAL REPORT  
ROCKY MOUNTAIN LABORATORIES MICROSCOPY BRANCH  
HAMILTON, MONTANA  
NATIONAL INSTITUTES OF ALLERGY AND INFECTIOUS DISEASES  
OCTOBER 1, 1993, TO SEPTEMBER 30, 1994

Fiscal Year 1994 saw a major reorganization of the Laboratory of Vectors and Pathogens into the Rocky Mountain Laboratories Microscopy Branch. However, a major research goal of the new branch remains to characterize in molecular detail those genes and gene products that are important in bacterial pathogenesis. This knowledge will then be exploited for the development of improved diagnostics and safe and effective, new generation vaccines. Dr. Claude F. Garon serves as Chief of the Rocky Mountain Laboratories Microscopy Branch.

The Bacterial Pathogenesis Section, under the direction of Dr. Claude F. Garon, continued its molecular dissection of the Lyme disease spirochete, *Borrelia burgdorferi*. Four outer surface proteins, OspA, OspB, OspC, and OspD, have been described for Lyme disease bacteria. OspA and OspB have been found to vary in molecular weight, isoelectric point, immunoreactivity, and sequence among the three spirochete species associated with Lyme disease. OspA and -B are cotranscribed and carried on a linear plasmid. The *ospD* gene has been mapped in some isolates to a 38 kb linear plasmid. Recently researchers have mapped the *ospC* gene to a 26 kb circular plasmid in the Lyme disease spirochete species. *ospC* represents the first *Borrelia* gene to be mapped to a circular plasmid. The cellular function of OspC and its potential contributions to the pathogenicity of the Lyme disease spirochetes are unknown. The immunological response of patients who had Lyme disease to this protein has been suggested to be more common in European than in North American patients. The immune response to OspC is primarily an immunoglobulin M response. Since the host immune response to OspC occurs early in the infection, it has been suggested that this protein may prove highly useful as an antigen for the serodiagnosis of Lyme disease. Researchers have been able to demonstrate that a protein with properties similar to those of OspC from Lyme disease spirochete species is carried by other species of the genus *Borrelia* including the relapsing fever spirochetes, *B. hermsii*, *B. parkeri*, and *B. turicatae*; the putative agent of epizootic bovine abortion, *B. coriaceae*, and *B. anserina*. This protein is referred to as an OspC homolog. In contrast to Lyme disease spirochetes, the *ospC* gene in other *Borrelia* species resides on linear plasmids. The transcriptional expression of this gene is most pronounced in some *B. hermsii* isolates. By primer extension analysis, it was noted that the *ospC* 5' untranslated leader sequence observed in the species investigated here has a length equivalent to that of the untranslated leader sequence of transcripts derived from the P1 *ospC* promoter in the Lyme disease spirochetes. Western blot (immunoblot) analysis with a polyclonal anti-OspC antibody provided further evidence for the expression of this gene and for the conservative nature of this protein among *Borrelia* species. The detection of this gene and its gene product in *Borrelia* species not associated with Lyme disease suggests that careful consideration must be given to the suggested use of OspC as an antigen for the serodiagnosis of Lyme disease. Analysis of the *ospD* gene has revealed that this gene is not universal among Lyme disease spirochete isolates. The gene was found to be carried by 90, 50, and 24% of the *B. garinii*, *B. afzelii*, and *B. burgdorferi* isolates tested. Size variability





in the *ospD*-encoding plasmid was also observed. Sequence analysis has demonstrated the presence of various numbers of a 17 basepair repeated sequence in the upstream control (promoter) region of the gene. In addition, a region within the coding sequence where various insertions, deletions, and direct repeats occur was identified. *ospD* gene sequences from 31 different isolates were determined and utilized in pairwise sequence comparisons and construction of a gene tree. These analyses suggest that the *ospD* gene was the target of several recombinational events and that the gene was recently acquired by Lyme disease spirochetes and laterally transferred between species.

The Facultative Intracellular Bacteria Unit, under the direction of Dr. Pamela L. C. Small, continued their studies on host-pathogen interactions. Investigation of the interaction of *Mycobacteria* with host cells has shown that *M. marinum*, like *M. tuberculosis* is capable of infecting and persisting within macrophage cell lines as well as within nonprofessional phagocyte cell lines. The optimal growth temperature of *M. marinum* is 32°C although organisms can be adapted to growth at 37°C. This fact has been used to explain the fact the *M. marinum* usually causes a granulomatous skin disease limited to the skin. However, systemic disease with *M. marinum* has been described in immunocompromised hosts. In order to determine whether virulence determinants were temperature regulated, strains were adapted to grow at 37°C. Investigators found that if a strain could be adapted to grow at 37°C as well as at 32°C, it would also be capable of persistence within macrophages. If a strain grew poorly at 37°C, it was unable to persist in macrophages. However, there was less cytotoxicity with bacteria grown at 37°C. Investigators have also isolated a *M. marinum* mutant which is unable to persist in macrophages. Researchers plan to use this strain as a background for isolating genes required for persistence in macrophages. Subcutaneous inoculation of *M. marinum* in a guinea pig produced granulomatous lesions. Early in infection, macrophages are filled with acid fast bacilli. After several weeks, a typical mycobacterial granuloma develops with Langerhans giant cells, epithelioid cells present and very few organisms. *M. ulcerans* like *M. marinum* causes persistent skin lesions in the human host. However, ulcerans lesions are characterized by extensive necrosis. Although these lesions may be extensive and deep, they are painless. Infection may persist for years with the development of satellite lesions. Necrosis is thought to be due to an uncharacterized cytotoxin. They have examined the interaction of *M. ulcerans* both with cultured cell lines and in a guinea pig model. *M. ulcerans* does not associate with nor enter nonphagocytic cells. This is particularly remarkable because even nonpathogenic mycobacteria such as *M. smegmatis* are capable of entry into cultured fibroblasts and epithelial cells. Furthermore, *M. ulcerans* are not taken up by cultured macrophage cells lines as readily as *M. marinum*. In contrast, *M. ulcerans* is cytotoxic to macrophage and fibroblast cell lines. This cytotoxic activity is present in sterile filtrates from *M. ulcerans* cultures. Researchers have found that subcutaneous injection of *M. ulcerans* into a guinea pig back produces necrotic lesions almost identical to those produced in humans. In contrast to *M. marinum*, *M. ulcerans* are not primarily intracellular. Instead the organisms appear as extracellular micro-colonies of acid fast bacilli lying next to the adipose cell layer. After several weeks, small granulomatous lesions form. It has generally been assumed that survival within the macrophage is responsible for the persistent nature of mycobacterial infections and yet intracellular survival is probably not a feature of infection with *M. ulcerans*. Although a cytotoxic activity has been reported in *M. tuberculosis*, a specific cytotoxin has not been identified. Researchers



plan to use genetic techniques to identify genes encoding the ulcerans cytotoxin and to determine whether these are present in *M. tuberculosis*.



## ADMINISTRATIVE

Drs. Willy Burgdorfer and John Munoz worked as scientist emeritus members of the laboratory during the year, providing valuable service and support.

Guest Researchers in Rocky Mountain Laboratories Microscopy Branch have included Dr. Stanley Falkow (Stanford University School of Medicine), Dr. Lucy Tompkins (Stanford University Hospital).

Joining the Laboratory as an IRTA Fellow during the year was Dr. Eldon Walker (University of Texas Medical School at Houston). Summer IRTA Appointments included Nicole Rintamaki, JoAnn Cloud, and Jennifer Maloney.

Visitors/Collaborators who spent varying amounts of time interacting with members of the RMLMB Scientific Staff included:

<u>Name</u>	<u>Affiliation</u>
Dr. Padmini Salgame	Albert Einstein College of Medicine, Bronx, New York
Dr. Pat Cleary	University of Minnesota, Minneapolis
Dr. Mike Minnock	Department of Biology, University of Montana, Missoula
Dr. Lu Barker	University of Missouri, Columbia
Dr. Lynn Miesel	Department of Biology, University of Utah, Salt Lake City
Dr. Tim Ryan	Neurology Department, Howard Hughes Institute, Stanford, California
Dr. Tone Tørjum	Bakteriologisk Institutt, National Hospital University of Oslo, Norway
Dr. Bruce Kohorn	Botany Department, Duke University, Durham North Carolina
Dr. Barbara Marsten	Emory University, Atlanta, Georgia
Dr. Kenneth Leigner	Intensive and Critical Care Medicine, Armonk, New York
Dr. Eldon Walker	Pathology Department, University of Texas Medical School, Houston, Texas

International Symposium of  
Bacterial Pathogenesis  
(Approximately 75 visitors)



## HONORS AND AWARDS

### **Dr. Claude F. Garon**

Served as Faculty Affiliate - Division of Biological Sciences, University of Montana

Served on Internal Advisory Committee, Electron Microscopy Facility, University of Montana

Served as Session Chairman for 7th Annual Lyme Disease Conference, Atlantic City, New Jersey

Served as Associate Editor, Journal of Spirochetal and Tick-Borne Diseases

Served as Editorial Board Member, Journal of Clinical Microbiology

### Invited Speaker

Division of Biological Sciences, University of Montana

Lyme disease 1994 State of the Art Conference, Stamford, Connecticut, April 22-23, 1994

Invited Presenter - Gordon Conference on the Biology of Spirochetes, Ventura, California January 1994.

### Reviewed Manuscripts

Journal of Clinical Microbiology

The Journal of Infectious Diseases

Infection and Immunity

Parasitology Today

Journal of Bacteriology

Journal of Spirochetal and Tick-Borne Diseases

International Journal of Systematic Bacteriology

Molecular Microbiology





**Dr. Willy Burgdorfer**

Invited Speaker

North California Lyme Disease Symposium, Ukiah, California, October 16, 1993.

San Francisco School of Medicine Symposium on the Epidemiology and Prevention of Infectious Diseases, San Francisco, California, April 2-4, 1994.

Third International conference on Diseases of the Alpine Region, Seis am Schlern, Italy, March 24-26, 1994.

Seventh Annual Borreliosis Conference, Stanford, Connecticut, April 22-23, 1994.

Honors

Honorary Degree, Doctor of Science conferred by Ohio State University, September 10, 1994.

Invited to serve as Associate Editor of the Journal of Spirochetal and Tick-Borne Diseases.

Scientific Director on the Board of Directors for the Lyme Disease Foundation.

Manuscripts reviewed

Journal of Wildlife Diseases

Acta Tropica

Journal of Medical Entomology

International Journal of Systematic Bacteriology

**Dr. Pam Small**

BioRad Training Course on Confocal Microscopy, San Francisco, California, May 10-13, 1994.

Grants reviewer for NIH.

Organized International Conference "Zen of Microbial Pathogenesis" at Rocky Mountain Laboratories, Hamilton, Montana, August 12-13, 1994.

Appointed Affiliate Faculty Division Biological Sciences, University of Montana, Missoula.

Invited Speaker

Keystone Conference on Bacterial Pathogenesis, Sante Fe, New Mexico, January 1-14, 1994.

University of Montana, Missoula, April 18, 1994.



Conference on Toxins and Pathogenesis, Andover, New Hampshire, July 17-22, 1994.

Moderator for Mycobacterial Section, ASM Western Meeting, Victoria, British Columbia, Canada, March 17-24, 1994.

Manuscripts reviewed

Journal of Bacteriology  
Gut

**Dr. David W. Dorward**

Invited Speaker

ASM Conference on Molecular Diagnostics and Therapeutics, Moran, Wyoming, September 26-30, 1993.

Lyme Disease Symposium, Ukiah, California, October 1993.

Sixth International Conference on Lyme Borreliosis, Bologna, Italy, June 19-23, 1994.

Lyme Disease Symposium, San Rafael, California, July 1994.

Meetings Attended

ASM General Meeting, Las Vegas, Nevada, May 23-27, 1994.

Manuscripts reviewed

Journal of Clinical Microbiology

**Dr. Scott Samuels**

Invited Speaker

Department of Microbiology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada, April 1994.

Seventh Annual Scientific Conference on Lyme Borreliosis, Stamford, Connecticut, April 1994.

Department of Biochemistry and Molecular Biology, New York Medical College, New York, April 1994.



Meetings Attended

Gordon Research Conference: Biology of Spirochetes. Ventura, California, January 1994.

Manuscripts Reviewed

Biochemistry

Grant Reviewed

International Science Foundation

**Dr. Richard Marconi**

Meetings Attended

Gordon Research Conference: Biology of Spirochetes. Ventura, California, January 1994.

**Dr. Lisa Pascopella**

Meetings Attended

Keystone Symposium on Molecular Events in Microbial Pathogenesis, Santa Fe, New Mexico, January 8-14, 1994.

**Dr. Scott Waterman**

Meetings Attended

Gordon Conference on Microbial Stress Response, Plymouth, New Hampshire, August 24-29, 1994



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AI 00488-08 RMMB															
PERIOD COVERED October 1, 1993, to September 30, 1994																	
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Ultrastructural Analysis of Antigenic Determinants in Pathogens																	
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Claude G. Garon</td> <td style="width: 33%;">Chief</td> <td style="width: 33%;">RMMB, NIAID</td> </tr> <tr> <td>OTHERS: W. Burgdorfer</td> <td>Scientist Emeritus</td> <td>RMMB, NIAID</td> </tr> <tr> <td>D. W. Dorward</td> <td>Sr Staff Fellow</td> <td>RMMB, NIAID</td> </tr> <tr> <td>S. F. Hayes</td> <td>Bio Lab Tech (Micro)</td> <td>RMMB, NIAID</td> </tr> <tr> <td>L. L. Lubke</td> <td>Microbiologist</td> <td>RMMB, NIAID</td> </tr> </table>			PI: Claude G. Garon	Chief	RMMB, NIAID	OTHERS: W. Burgdorfer	Scientist Emeritus	RMMB, NIAID	D. W. Dorward	Sr Staff Fellow	RMMB, NIAID	S. F. Hayes	Bio Lab Tech (Micro)	RMMB, NIAID	L. L. Lubke	Microbiologist	RMMB, NIAID
PI: Claude G. Garon	Chief	RMMB, NIAID															
OTHERS: W. Burgdorfer	Scientist Emeritus	RMMB, NIAID															
D. W. Dorward	Sr Staff Fellow	RMMB, NIAID															
S. F. Hayes	Bio Lab Tech (Micro)	RMMB, NIAID															
L. L. Lubke	Microbiologist	RMMB, NIAID															
COOPERATING UNITS (if any) LPVD, RML, NIAID; LMSF, RML, NIAID; LICP, RML, NIAID; Department of Microbiology, Stanford University School of Medicine; Department of Microbiology and Immunology, University of Rochester Medical Center																	
LAB/BRANCH Rocky Mountain Laboratories Microscopy Branch																	
SECTION Bacterial Pathogenesis																	
INSTITUTE AND LOCATION NIAID, NIH, Bethesda, MD																	
TOTAL MAN-YEARS: 2.7	PROFESSIONAL: 1.2	OTHER: 1.5															
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) Human subjects</td> <td><input type="checkbox"/> (b) Human tissues</td> <td><input checked="" type="checkbox"/> (c) Neither</td> </tr> <tr> <td><input type="checkbox"/> (a1) Minors</td> <td></td> <td></td> </tr> <tr> <td><input type="checkbox"/> (a2) Interviews</td> <td></td> <td></td> </tr> </table>			<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither	<input type="checkbox"/> (a1) Minors			<input type="checkbox"/> (a2) Interviews								
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither															
<input type="checkbox"/> (a1) Minors																	
<input type="checkbox"/> (a2) Interviews																	
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>             Researchers in the laboratory have been able to demonstrate that extracellular components of <i>Borrelia burgdorferi</i> 1) appear to be present wherever active growth of the organism is taking place and therefore may be useful as a diagnostic indicator of active infection and/or treatment effectiveness; 2) are involved in the packaging and protection of intact DNA molecules containing a few known and many unknown genes and gene products; 3) appear to specifically interact with immunoglobulin M molecules in a unique fashion, perhaps to escape immune surveillance; and 4) possesses potent, non-specific mitogenic activity which may cause an inappropriate and noneffective stimulation of the immune system triggering autoimmune disease components. Furthermore, experiments suggest that <i>B. burgdorferi</i> can utilize transferrin as a source of ferric ions for growth in a process that involves direct interaction with an inducible surface receptor. Researchers continue to examine these and other bioproducts with the aim of improving the prevention, treatment and diagnosis of Lyme disease.           </p>																	





## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 AI 00554-06 RMMB

## PERIOD COVERED

October 1, 1993, to September 30, 1994

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural Characterization of Microbial Genes and Nucleic Acid Molecules

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Claude F. Garon	Chief	RMMB, NIAID
OTHERS:	R. T. Marconi	IRTA Fellow	RMMB, NIAID
	D. W. Dorward	Sr Staff Fellow	RMMB, NIAID
	D. S. Samuels	IRTA Fellow	RMMB, NIAID
	L. L. Lubke	Microbiologist	RMMB, NIAID
	V. E. Tamplin	Microbiologist	RMMB, NIAID

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Rocky Mountain Laboratories Microscopy Branch

## SECTION

Bacterial Pathogenesis

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

4.3

## PROFESSIONAL:

2.8

## OTHER:

1.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Lyme borreliosis is now the most common arthropod-borne disease in the United States. *Borrelia burgdorferi*, the causative agent, has been isolated from humans, mammals, birds and arthropods and is cultivable in the laboratory. *B. burgdorferi* has an unusual genome composed of a linear chromosome of about 950 kb and variable numbers of linear and circular plasmids that range in size from about 5 to 60 kb. The linear and circular plasmids have copy numbers of about one per chromosome. Some of these plasmids are lost during *in vitro* cultivation. The linear plasmids have covalently closed hairpin ends with structures and sequences similar to those of African swine fever virus telomeres. Only four genes have been mapped currently to plasmids; the remainder are on the chromosome. The genes for the major outer-surface proteins OspA and OspB are on a 49 kb linear plasmid in B31 and on a slightly larger plasmid in strains ACAI and R-IP90. The gene for OspD is on a 38 kb linear plasmid in B31 that is often lost upon *in vitro* cultivation. The gene for OspC, a major immunodominant protein, is on a 26 kb circular plasmid in all three Lyme disease species and therefore represents the first *Borrelia* gene mapped to a circular DNA molecule. The objective of this project, therefore, is to define the genetic capacity of *B. burgdorferi* and other vector-borne pathogens in sufficient detail to begin the process of mapping those genes and gene products which may be important in pathogenesis.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AI 00670-02 RMMB

## PERIOD COVERED

October 1, 1993, to September 30, 1994

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Genetic and Physiological Basis of Acid Resistance in Enteric Bacteria

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P.L.C. Small	Sr Staff Fellow	RMMB, NIAID
OTHERS:	Scott Waterman	Visiting Fellow	RMMB, NIAID
	Diane Welty	Microbiologist	RMMB, NIAID

## COOPERATING UNITS (if any)

Dr. Joan Slonczewski, Department of Biology, Kenyon College, Gambier, Ohio

## LAB/BRANCH

Rocky Mountain Laboratories Microscopy Branch

## SECTION

Facultative Intracellular Bacterial Unit

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

.74

## PROFESSIONAL:

.58

## OTHER:

.16

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

*Shigella* species, the causative agents of bacillary dysentery, are unusually infectious. Volunteer studies have shown as few as 10 ingested organisms are sufficient to produce disease. This is particularly remarkable when one considers the fact that the inoculum must pass through the stomach, with a pH of less than 3.0 in order to reach to intestines where entry into and replication within colonic epithelial cells results in dysentery. Enteric pathogens such as *Salmonella* species and *Vibrio cholera* require an infectious dose over 10,000-fold greater than that of *Shigella* in order to cause enteric disease. *Shigella* is able to survive exposure to pH 2.5 for several hours whereas *Salmonella* species are extremely acid sensitive. The acid sensitivity of oral *Salmonella*-based vaccine strains is a major impediment to their utilization.

The objectives of this study are to characterize the genetic and physiologic basis of acid resistance in *Shigella* species and to utilize this information to construct more efficacious oral vaccine strains. We have identified a gene from *Shigella flexneri*, *rpo<sup>s</sup>*, which confers acid resistance on an acid sensitive *Escherichia coli*, HB101. *Rpo<sup>s</sup>* encodes a stationary phase sigma factor which acts indirectly in conferring acid resistance on *Shigella* species. In order to identify specific genes required for acid resistance, 36 acid sensitive mutants were obtained using transposon mutagenesis. Three of these classes of mutations contain insertions in genes with known homologies: *PhoN Salmonella typhimurium*, *MxiA*, a plasmid encoded gene required for virulence in *S. flexneri*, and *OmpT*, a protease in *E. coli*. The remaining six classes of insertions fall within genes for which there is no significant DNA homology with known genes. The expression of some of these acid-resistance genes appears to be regulated by *rpo<sup>s</sup>*. Identification and further analysis of this set of acid resistant genes will be useful in the construction of acid resistant oral vaccine strains.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AI 00671-02 RMMB

## PERIOD COVERED

October 1, 1993, to September 30, 1994

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Molecular and Cell Biology of Mycobacterial Species

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Pam L. C. Small	Sr Staff Fellow	RMMB, NIAID
OTHERS:	Lisa Pascopella	IRTA Fellow	RMMB, NIAID
	Stanley Falkow	Collaborating Scientist	Stanford University
	Diane Welty	Microbiologist	RMMB, NIAID

## COOPERATING UNITS (if any)

Raphael Valdivia, Stanford University, Stanford, California; Lalita Ramakrishnan, Stanford University, Stanford, California; Tone Tønjum, National Hospital, Oslo, Norway; Richard Wyatt, NIH, Bethesda, Maryland

## LAB/BRANCH

Rocky Mountain Laboratories Microscopy Branch

## SECTION

Facultative Intracellular Bacteria Unit

## INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

.96

## PROFESSIONAL:

.91

## OTHER:

.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The re-emergence of tuberculosis has led to renewed interest in facultative intracellular pathogen, *Mycobacterium tuberculosis*. Soon after entering the host, the *Mycobacterium* is engulfed by macrophages. This encounter between bacterium and macrophage determines the outcome of the pathogen-host interaction. If bacteria are able to resist killing by the macrophage, they will remain in the host for decades, re-emerging to cause disease when the host immune system is compromised by old age, HIV infection, or chemotherapy. How mycobacterial pathogens are able to persist within the host for decades is unknown but it is believed they are in a non-replicative, dormant state. Our goal is to study the fate of *Mycobacterium* within the host cell, to characterize cellular and bacterial components required for bacterial entry into and survival within host cells, and to identify bacterial components required for persistence in the host. We are using *M. marinum* and *M. ulcerans*, both of which cause persistent disease in humans, as model systems for studying *M. tuberculosis*. We have shown that these two species are more closely related to *M. tuberculosis* than any mycobacterial pathogen aside from *M. bovis*. Our studies show that *M. marinum*, like *M. tuberculosis*, replicates and persists within macrophages. We have isolated a *M. marinum* derivative which has lost the ability to persist in host cells and are using this strain in complementation studies to identify components from *M. marinum* required for persistence and will determine whether they are present in *M. tuberculosis* as well. Many bacterial species contain a stationary phase sigma factor which turns on genes transcribed in dormant bacteria. We have identified a homologous sigma factor from *M. tuberculosis*, *M. marinum*, *M. ulcerans*, and *M. haemophilum*. Investigations are in progress to identify the role this gene may play in the ability of mycobacteria to persist within host cells. We found cloned mycobacterial DNA can be electroporated into *M. marinum* and that plasmid vectors which replicate in *M. tuberculosis* are stably maintained in *M. marinum*. *M. marinum* replicates much more rapidly than *M. tuberculosis* which makes this system invaluable in studying the genetics of mycobacterial virulence.













<http://nihlibrary.nih.gov>

10 Center Drive  
Bethesda, MD 20892-1150  
301-496-1080





